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Review Article

Microsporidiosis in the Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae)

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ABSTRACT

The mulberry silkworm, *Bombyx mori* L., is prone to infection of various pathogenic organisms. Microsporidiosis of the silkworm, caused by highly virulent parasitic microsporidian or *Nosema bombycis* (Nageli), is one of the most serious maladies, which determines the success or failure of sericulture industry in any country. Infections of the disease ranging from chronic to highly virulent can result in heavy loss to the sericulture industry. Several strains and species of microsporidia have since been isolated from the infected silkworms, and the disease is becoming increasingly more and more complex. Epizootiology, development of immunodiagnostic kit, fluorescent antibody technique and use of ideal disinfectant, chemotherapy and thermo-therapy techniques and management strategies have been addressed for identification, destruction, prevention and control of disease causing micro-organisms. Techniques of forced eclosion test and delayed mother moth examination have also been stated to play important roles in the detection of the disease. An attempt has also been made in this review article to briefly elucidate the various aspects of the pebrine disease and to help the researchers to develop efficient model(s) for the prevention, control and management of microsporidia infecting mulberry silkworm, *Bombyx mori* L.

Keywords: Bombyx mori L., Microsporidiosis, Pathogenesis

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INTRODUCTION

The microsporidia are spore forming, small, obligate, intracellular living eukaryote infecting both beneficial and non-beneficial insects (Nataraju *et al*., 2005). More

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than 140 genera and 1200 species of microsporidia have been recorded from insects and fish (Canning, 1993; Samson *et al.,* 1999a). Among these, at least 200 belong to the genus *Nosema* (Sprague, 1982) and most *Nosema* species are parasitic to invertebrates. A majority of these, including *N. bombycis* and *N. tyriae* (Canning *et al*., 1999), *N. mesnili* (Cheung & Wang, 1995), *N. algerae* (Muller *et al*., 2000), *N. aphis* and *N. trichoplusiae* (Malone *et al*., 1994) are pathogenic to various insects. The microsporidian infection remains a major threat to the sericulture industry with its recurrent occurrence. More than twenty wild insect species have been found to have microsporidian spores that can cross-infect silkworm. Pebrine, i.e. the spores of microsporidian (*Nosema bombycis*), is one of the most dreaded diseases of the silkworm, *Bombyx mori.* Pebrine, which determines success or failure of the sericulture industry of a nation, infects almost all ages, stages, breeds and hybrids of the silkworm by both transovarial and peroral infections. It is highly infectious and difficult to eradicate after the occurrence of infection. This is evidenced from the historical fact that the rise and fall of pebrine disease correspond with the ups and downs of the sericulture industry in the silk producing countries of the world (Tatsuke, 1971). The earliest research on pebrine was confined especially with the epizootiology and prevention of the disease (Fujiwara, 1979; Ishihara, 1963; Weiser, 1969). Meanwhile, the microscopical method of mother moth examination, although widely practiced mainly due to its simplicity, does not assure a foolproof detection of the microsporidian.

To circumvent this particular problem, efforts have been made to evolve simple, precise and more accurate method to detect the disease (Baig *et al.,* 1992; Fujiwara, 1993; Geethabai *et al.,* 1985; Shi & Jin, 1997), identify alternate host (Fujiwara, 1993; Samson, 2000), use chemotherapy and thermo-therapy for the prevention and control of disease (Hayasaka, 1990), apart from the identification of intermediary stages (Santha *et al*., 2001) but with little success. Even though research and fight against the pebrine have been continuously done for more than a century, loss due to the disease has not been completely eliminated (Singh *et al*., 2010). However, historical evidences suggest a significant relationship between the success of sericulture industry and the control of the disease. Therefore, to improve the sericulture industry and to save it from crop losses due to this chronic disease, it is essential to have a foolproof diagnostic and preventive technique.

To briefly review and discuss the recent advances achieved so far on the various aspects of the pebrine disease, an attempt has been made in this article to present annotated information on the causative organism, pathogenesis, manifestation, diagnosis and management of microsporidia infecting mulberry silkworm, *Bombyx mori,* in order to develop efficient model(s) for the prevention and control of this particular chronic disease in the days to come.

Disease History

Several historical evidences in various countries of the world showed that the outbreak of pebrine disease had greatly influenced the decline of the sericulture industry in the past. The damage of crops in Europe in the middle of the 19th century was so great and extensive which became a worldwide scale that the cocoon production sharply declined and the sericulture industry of the world suffered heavily (Tatsuke, 1971). The history of research on the pebrine disease progressed with the advancement of microbiology in the 19th century. The disease-causing microorganism was first observed in the haemolymph of the silkworm and was given the name 'Hematozoid' (Guerin-Menevillae, 1849). Quadrefague (1860) coined the name pebrine because of the appearance of pepper-like spots in the diseased larvae. Nageli (1857) of Germany stated that the disease is caused by a protozoan parasite and named this pathogen as *Nosema bombycis*. The noted French microbiologist, Pasteur (1870), in his book entitled *"Etudes sur la maladie des vers a Soie"*, called the disease 'corpuscle disease' and made a detailed study on its growth and transmission, and discovered that the disease is transmitted through transovarian transmission within the body of the mother moth and suggested methods of preventing the disease.

In India, the first record of the spread of incidence of disease was made at the end of the 19th century in the Kashmir valley (Sahaf, 2002). In 1890-1900, the disease swept through Mysore and Madras provinces. Thereafter, the disease reappeared during 1925-1930 in an epizootic form (Chitra *et al*., 1975). Disease epidemics were again observed during 1991-1992 in the southern part of the country, which resulted in considerable crop losses and revenue (Nataraju & Dandin, 2006). Since then, the incidence of the disease has been observed intermittently in silkworm crops in the different parts of India. The pebrine incidence also caused a considerable loss of silkworm seed during 1997-1999 in the seed production area of Uttar Pradesh (Quadri & Khatri, 2005).

Life cycle of Nosema bombycis

On the basis of morphological and molecular features, Undeen and Cockburn (1989) and Vossbrinck *et al*. (1987) stated that *N. bombycis* is one of the earliest known primitive eukaryotes because of the primitive type of nuclear division, but it lacks some typical organelles mainly mitochondria, stacked golgi, prokaryotic sized ribosomes and ribosomal RNAs. Several workers have studied the developmental cycle of *N. bombycis* and presented a comprehensive account of the life history of the disease (Iwano & Ishihara, 1981; Kawarabata & Ishihara, 1984). The life cycle of *N. bombycis* includes three stages, namely, spore, planont and meront. The mature spore is oval or ovo-cylindrical and measures approximately 3.4 -3.8 µm in length and 2.0 - 2.3 µm in width, with three-layered membrane (inner, middle and outer). The spores are highly refractive, and shine bluish white under microscope exhibiting

'Brownian movement'. The outline is smooth and the spores are heavier than water. The resistant form of the disease is spore and it remains either in an infected tissue of the body or discharged through excreta by leaving infected host tissue. The spore, when swallowed by the silkworm through contaminated food, germinates under alkaline conditions inside the gut of host with the help of digestive juice and produces a long polar filament measuring 500 µm in length and 0.5 µm in width, and it is more than 30 times longer than that of the lengthwise dimension of the spore, on the end of which grows a sporoplasm (Peter *et al.,* 1999). The sporoplasm has one or two nuclei and other cell organs and possesses limited membrane. The sporoplasm multiplies through fission, comes out of haemolymph through intracellular spaces, spreads to every part of the body, lives in various systems (particularly in body fat and muscular tissue) and becomes nucleus to form spore after multiplication through fission (Abe & Fujiwara, 1979). Formation of spore is apansporoblastic, disporous and dimorphic. One type of the sporoblast of the long polar tube types turns into a single spore with many coils of polar tube. The other type of sporoblast, with short polar tube, turns into a single spore with a few coils of the polar tube. Spore with short polar tube hatches directly in the host cell. Secondary sporoplasm reaches the other cells of the host. The spore completes its life cycle within 4 days. Complete developmental stages of the pathogen have been studied and elucidated in detail (Takizawa *et al.,* 1975). The mature spore is unicellular endo-membranous differentiation of its sporoblast (Vavra & Maddox, 1976). These authors designated the sporoblasts as Phase-I sporoblasts and Phase-II sporoblasts. The Phase-I sporoblasts are characterized by the presence of a dark staining spherical body (Singh *et al*., 2007).

Characteristics of the Disease

The disease infects all ages, stages, breeds and hybrids of the silkworm. Larvae suffering from pebrine do not show any external symptoms until the disease is far advanced. At advanced stage, larvae become sluggish and show symptoms like poor appetite, retarded growth and development and irregular moulting. As the disease progresses, the larvae appear pale, dull and translucent with wrinkled skin, shrink in size and become flaccid (Jolly, 1986; Singh & Saratchandra, 2003). Due to the chronic nature of the disease, the infected larvae do not die immediately and continue to survive for some time. The infected gut becomes opaque and white pustules appear on the silk glands. Infected pupae are flabby and swollen with lusterless, blackish and softened abdomen and black spots occasionally appear on this region (Ishihara, 1963). Highly infected pupae fail to metamorphose into adult. Irregular moth emergence, clubbed wings, distorted antennae, improper mating, low fecundity, and sometimes clumpy egg laying, as well as high percentage of unfertilized and dead eggs, apart from eggs with less gluey substance leading to their detachment from the egg sheets, lack of uniformity in egg

shape, and easily coming off scales from the wings and abdominal area are some of the symptoms of the disease at the moth stage. The accessory glands of pebrinized moths are also infected and this results in production of loose eggs which easily role-off the egg sheets.

Source and Stage of Contamination

Transovarially infected seeds are the primary source of contamination. Contaminated rearing and grainage buildings, appliances, silkworm litter, and mulberry leaf fed to the silkworm harboured by infected insects, *etc.* also contribute to the spread of the disease. The incidence of pebrine varies with the variety of silkworms, the developmental stage and the rearing environment. Meanwhile, resistance to pebrine is greater in the Chinese breeds, but less in the Japanese and the least in European breeds (Govindan *et al.,* 1998). The multivoltine breeds are relatively more resistant than bivoltines (Patil & Geethabai, 1989). Young silkworms, newly moulted and starving larvae are susceptible and show high mortality. In India, Nistari and C. Nichi are more resistant silkworm breeds as compared to the others. Patil and Geethabai (1989) reported that among the bivoltines breeds, NB7 is the most susceptible, and this is followed by NB4D2, KA and NB18. Although the disease resistance appears to depend on the genetic constituents of a particular breed, factors such as pathogen load, inadequate nutrition, and the environment in which the insects are reared may also affect their resistance. In addition, the physical and physiological characteristics of the hosts may make the invasion of microsporidians possible (Weiser, 1969, 1977). The larvae infected during the 1st and 2nd instars show a normal growth up to the 3rd instars. Meanwhile, disease symptoms appear during the later half of the 4th instars to the first half of the 5th instars and die before spinning. If the contamination takes place in the 3rd instars, the larvae will show symptoms of the disease in the late 5th instars and die on the mountage before cocooning. It is important to note that these larvae discharge spores through faecal matter during the 4th and 5th instars. If these larvae are reared with healthy larvae, the spore discharge by the infected larvae provides the source of contamination and digestion of spores by healthy silkworms will result in a spread of the disease. This stage of contamination is known as the 'second stage of contamination.' Larvae infected during the 4th and 5th instars pupate and on emergence lay contaminated eggs. This phenomenon is referred to as 'transovarian transmission.' Most of the larvae infected through transovarian transmission show irregular moulting and growth, become tiny or under grown and die before the 3rd moult, after discharging spores. The contamination occurring from the transovarially-infected larvae is termed as the 'first stage of contamination.' The minimum number of the spores required for contamination through per oral infection varies with each instar. Among other, Iwano and Ishihara (1981) stated that 1-10 spores are sufficient enough to cause disease in

the 2nd instars larvae, while approximately 100 of such spores are required in the 5th instars for the same symptoms to occur. Transovarian transmission is 100% in the case of *N. bombycis* and only 1.2% with *Nosema* sp. M11 (Han & Watanabe, 1988).

The spores of different microsporidia infecting silkworms differ in their morphological characters; some are larger than mature spore and some are long, thin and pear-shaped with different sizes, shapes and lusters. Sometimes, the conidia of green muscardine and red muscardine bear a striking resemblance to the spore of the pebrine disease. Horizontal transmission of the pebrine spore is possible through contaminated rearing bed, mulberry leaf and layings (Govindan *et al.,* 1998). Baig *et al.* (1988a) reported that the spread of disease in rearing trays is also dependent on the density of diseased silkworms. Growth and multiplication of pathogen are influenced by the growth of its host. When egg enters into diapause, the growth and multiplication of pathogen stops simultaneously and when egg starts to grow, the pathogen will also start to grow and multiply.

Physiological Stability

Generally, a large number of factors, *viz.,* temperature, humidity and abiotic components of the substrate influence the survival of microsporidians (Kramer, 1976). The spores belonging to the dormant stage of pathogen and possessing great resistance can remain infective after 3 years in the dried body of the female moth, and become active after being submerged in water for 5 months (Li, 1985). When kept in the dark, the spores are reported to remain viable for as long as seven years, but when the spores are directly exposed to sunshine, they remain viable for 6-7 hrs and when treated with hot water, they survive for just 5 minutes. Studies conducted on the viability of the pebrine spores in soil and compost under tropical conditions have shown the survival of spores for a maximum period of 225 days in wet soil and a minimum of 135 days in wet compost (Patil, 1993). Srikanta (1986) observed that spores remained infective even after 150 days of refrigeration and after 90 days in moist soil and faeces. He further stated that the viability of spores is lost in 60 days in dry soil and in 5 days when they are stored at room temperature. The resistance of spores to different disinfectants indicates that they can remain viable for 10-30 minutes in the solution of corrosive sublimate, for about 5 hrs in formalin and 10 hrs in chlorinated lime solution (diluted 10,000 times). Bleaching powder containing 1% and 3% active chlorine can render spore inactive in 30 minutes and 10 minutes, respectively. When the degree of infection is relatively high, the egg often becomes sterile or dead, but when the contamination is of low degree, the egg hatches and the disease develops at the larval stage and causes death of larvae at later stages of development.

Alternate Host

Most microsporidians prefer having alternate hosts because these offer many advantages to them, *viz.*, dispersal, transmission and

survival. The perpetual incidence of microsporidian infection in silkworms may be due to various sources of secondary contaminations which include alternate hosts in and around mulberry garden. In addition to *N. bombycis,* seven other microsporidia belonging to the genera *Nosema, Pleistophora, Thelohania, Vairomorpha* and *Leptomonas* spp. have been isolated from the silk moth (Govindan *et al.,* 1998). They differ in their spore morphology, target tissues and virulence, and have been designated as M11, M12 and M14 (*Nosema* sp.), M24, M25, M27 *(Pleistophora* sp.) (Fujiwara, 1984a and b) and M32 (*Thelohania* sp.) (Fujiwara, 1985), as shown in Table 1. Three microsporidia designated as NIK-2r, NIK-3h and NIK-4M have been isolated from silkworms in Karnataka (India) and these are immunologically dissimilar to *N. bombycis* (Ananthalakshmi *et al*., 1994).

N. bombycis has also been reported to infect *Samia cynthia ricini* and Indian tropical tasar, muga and Chinese tasar silkworms (Talukdar, 1980). *N. bombycis* has also been found to infect several other lepidopteron like *Spodoptera exigua*, *S. litura*, *Diaphania pulvurentalis*, *Pieris rapae, P. brassicae, etc.* Veber (1958) reported 32 species of lepidopteron which are known to develop infection to the peroral inoculation of *N. bombycis* spores. These include *Chilo suppressalis, Pieris rapae, P. brassicae, Spodoptera exigua*, *S. litura, S. maurilia, Balataea funeralis, Cruptophlebia illepida, Exartema mori, E. morivirum, Diaphania pyloalis, Mycalesis gotoma, Abracus miranda, Descorba simplex, Boarmia selenlia, Menophra atrilinecta, Elydna nonagrica, Otosema odera, Perigea illecta, Plusia chalcites, Pseudaletia unipuncta, Stilpnotia lubricipeda, S. imparilis, Callimorpha quadripunctata, Thaumetopoea processionea, Malacosoma neustria, Gastropacha quercifolia, Lasiocampa quercus, Bombyx mandarina, Antheraea pernyi, A. yamamai, Sphinx ligustris, Agrotis ipsilon, Agrius cinagulatus, Pholera assimilis, Acronicta major,*

TABLE 1
Different types of microsporidian spores

Types of microsporidian	Spore size (µm) (L x W)	Site of infection	Virulence
Nosema bombycis	3.8 x 2.2	Systemic	High
Nosema sp. (M11)	3.9 x 1.9	Various tissues	Low
Nosema sp. (M12)	4.2 x 2.7	Various tissues	Low
Nosema sp. (M14)	5.1 x 2.0	Various tissues	High
Pleistophora sp. (M24)	2.7 x 1.6	Mid gut	Low
Pleistophora sp. (M25)	3.2 x 1.8	Mid gut	Low
Pleistophora sp. (M27)	5.4 x 3.0	Various tissues	Low
Thelohania sp. (M32)	3.4 x 1.7	Muscle	Low

Source: Fujiwara (1985)

Acrotomycis aceris and *Achaea janata* (Kawarabata, 2003; Samson *et al.,* 1999b; Singh *et al*., 2007, 2010). The lawn grass cut worn, *Spodoptera depravata,* serves as a natural reservoir for the pathogen (Ishihara & Iwano, 1991) which shares the surface specific antigens with *N. bombycis and* results in transovarial transmission with less virulence.

Cross Infectivity

Different species of insects, known to carry microsporidians causing cross-infectivity to silkworms, were found harbouring in and around mulberry garden. Enormous quantity of microsporidian spores was observed in *Catopsilia* sp., an inhabitant of mulberry garden (Kishore *et al*., 1994) and found infective to silkworms. Butterflies causing microsporidian infections to silkworms were also reported (Samson *et al*., 1999a, b). Singh *et al.* (2007) reported that butterflies, i.e. *Eurena hecabae* and *Zizina otis*, carry microsporidian spores infective to silkworms. These insects are potential source of contamination as spores of pathogen are excreted along with litter on the mulberry leaves in the garden, and when these leaves are fed to silkworms, they cause the disease to appear. The different microporidia isolated relatively recently from India, their spore morphology, target tissues, as well as the infection rate and rate of transovarian transmission in progeny of silkworm are presented in Table 2, which reveals the transmission to the extent of 100% in NIK-3r, whereby only 1.8% is found in NIK-3h.

Spore Isolation and Purification

Isolation, purification and identification of spores from the host are the first steps involved in the study of pebrine disease and its management. To isolate the spores, diseased larvae/pupae/moths are homogenized in sterile water for 1-2

TABLE 2
Microsporidian spores isolated from India

Characters	Microsporidian spores		
	Nosema sp. NIK-2r	*Nosema* sp. NIK-3h	*Nosema* sp NIK-4m
Spore size (µm) (L x w)	3.6 x 2.8	3.8 x 1.8	5.0 x 2.1
Spore shape	~ round	Oval	Ovocylindrical
Site of infection	Gut epithelium, malpighian tube, muscle, fat body, silk gland, gonad	Malpighian tube, muscle, fat body, silk gland, gonad	Gut epithelium
Infection rate	High	Medium	High
Mortality rate	High	Low	High
Rate of transovarian transmission of spores in progeny	100%	1.8%	No report

Source: Nataraju *et al.* (2005)

minute(s) using a mixer. The homogenate is filtered through cotton or fine muslin cloth. The filtrate obtained is transferred into a centrifuge tube and is centrifuged at 3,000 rpm for 5 minutes. The supernatant is discarded and the sediments obtained consist mostly of spores, which can be confirmed with microscopical examination. However, serological and biochemical studies of microsporidians require high degree of purity. Gochnaner and Margetts (1980) described a rapid method for concentrating *Nosema* spores based on continuous flow centrifugation method. Another method based on 'Brownian movement' was also reported. Sato and Watanabe (1980) purified spores using sucrose and percol gradient centrifugation and reported that centrifugation using percol at 73,000g for 30 minutes resulted in 3 bands viz., a sharp band consisting of tissues of silkworms, mulberry leaves, bacteria, etc., a dim band consisting of mature but inactive spores and sharp band consisting of only mature and active spores.

Sporulation Rate

Sporulation rate is a significant step in the area of pebrine disease detection through microscopic test. In this method, the mother moths are collected in groups after oviposition and in perforated cardboard boxes/covers and preserved alive. Alternatively, they can be left on dummy sheets in the oviposition trays itself. The boxes/oviposition trays are properly numbered as per egg sheets and preserved in well-ventilated room at ambient room temperature (25-30^{0}C) for a period of 3-4 days prior to the microscopic test. This enhances sporulation of the pathogen in older moths facilitating an easy and more accurate detection of the disease. After the stipulated period, moth testing was carried out as per recommended procedure in-vogue. Through this method, easy and effective detection of pebrine disease is possible due to enhanced sporulation in older moths. Even under moderately low infection levels, pebrine can be detected using this method. This technique is very useful during basic seed multiplication and production of P1 seeds. It has also been reported that the rate of multiplication of *N. bombycis* increases substantially with the age of moths and the cephalothoraxic region has the highest spore concentration, especially around the wing and wing muscles (Sashidharan *et al*., 1994) (Table 3), and therefore, testing of silk moths 3-4 days after oviposition would be a more effective method to detect pebrine with better accuracy.

Approaches for Prevention and Management of Pebrine

Pebrine has been a threat to the sericulture industry since time immemorial. The disease has become more complex now because of the occurrence of the different types of microsporidians infecting the silkworm. Some of them belong to other genera like *Vairomorpha* and *Thelohania* and exhibit differences in their patterns of infection (Samson, 2000). Apparently, the biology of the pathogen has been used

TABLE 3
Sporulation rate of *Nosema bombycis* in different tissues after emergence of moths of silkworm

Body parts	Breeds	Quantity of spores on different hours after emergence ($x10^7$/gm wt of tissue)				
		0 h	24 h	48 h	72 h	96 h
Whole moth	PM	4.39	4.50	5.67	21.90	25.50
	NB18	5.92	6.34	12.40	22.00	28.70
Cephalothorax	PM	8.20	10.50	9.40	35.80	44.00
	NB18	7.10	10.20	14.70	38.40	40.10
Abdomen	PM	1.49	2.60	5.50	14.90	21.60
	NB18	5.02	3.80	7.94	14.00	20.30
Wing	PM	6.30	8.50	11.00	25.00	31.30
	NB18	8.61	12.80	24.45	28.60	34.60
Gut	PM	9.62	10.81	10.60	24.60	22.60
	NB18	8.11	8.94	12.40	20.00	21.20
Fat body	PM	0.19	0.15	0.10	0.20	0.20
	NB18	1.34	2.17	2.10	1.77	2.41

Source: Sashidharan *et al*. (1994)

as a basis in disease control. The disease is transmitted horizontally by ingestion of spore and vertically by transovarian transmission. This unique characteristic of the disease makes it difficult to be completely eliminated from the silkworm crops. The earliest method suggested by Pasteur, based on the selection of pathogen free eggs through a careful systematic examination of mother moths for pathogens after laying eggs, is one of the most effective methods to avoid the disease in the silkworm crops.

Meanwhile, proper monitoring and testing of the seed crops at every successive stage of progress of the crop are done to ensure the production of pebrine free seed cocoons for commercial seed production. Quadri and Khatri (2005) stated a three-tier examination approach (namely, larval, pupal and moth) to detect the incidence of pebrine disease in the multiplication of silkworm seed and suggested destruction of infected crops as soon as identification of infection as an important step towards pebrine disease management. Since the disease is seed borne, the surface sterilization of eggs immediately after egg laying and also during the pin-head stage of incubation should be followed to prevent the occurrence of the disease from surface contamination (Singh *et al.,* 1992). Several reports have documented the efficiency of the thermal treatment of silkworm eggs in minimizing pebrine infection (Bedniakova & Vereiskava, 1958; Fujiwara & Kagawa, 1984; Hayasaka, 1990). The maximum lowering of infection rate was reported in eggs incubated during the first two days of their development to 44°C. Singh and Saratchandra (2003)

stated that the incubation of eggs at higher temperature within 3 days of laying would result in significant reduction in pebrine disease. Meanwhile, thermal treatment, in combination with hydrochlorization to achieve dual objectives of elimination of pebrine and termination of diapause, has also been reported (Austrurov *et al.,* 1969). Liu *et al.* (1971) reported a remarkable success in reducing pebrine infection after a treatment at 47°C for 10-20 minutes. Chowdhary (1967) suggested exposure of cocoons to high temperature (33.8°C) at the time of pupation for 16 hrs a day, whereas 55 - 65% of humidity tends to reduce infection in the resulting eggs. Sheeba *et al* (1999) reported that a thermo-therapy of 7 days old pebrinized cocoons at 36°C for 16 h tended to significantly reduce pebrine infection without affecting the growth and development of the larvae.

Certain insect hosts tolerate high temperature than their microsporidian parasites and the hosts can be freed of the disease by rearing the infected individuals at higher temperatures until the disease is cured. Meanwhile, attempts have been made by several authors/researchers to control pebrine infection in silkworm eggs through temperature treatment. Among other, Ovanesyan and Lobzhanidze (1960) and Austrurov *et al.* (1969) attempted hot water treatment of pebrinized eggs and reported a sharp decrease in the degree of infection. Similarly, Smyk (1959) expressed varying successes with hot water treatment. Fujiwara and Kagawa (1984) reported that the parasites in non-diapausing eggs were more sensitive to hot water (46°C for 4 minutes) treatment and there was no harmful effect of the treatment on the normal development of silkworm embryos. The treatment of silkworm eggs, with HCL of 1.03 - 1.09 specific gravity at 47^0C for 10-20 minutes, has been known to reduce the disease incidence by 97.4 - 100% (Liu & Zhong, 1988). In the same manner, a hot air treatment (48-50^0C) of 12-18 h old silkworm eggs also inhibited the development of microsporidians. Silkworm eggs of 36-60 h old treated with hot water at 46^0C for 90-150 minutes, 48^0C for 50-70 minutes, and 52^0C for 4 minutes, also inhibited the development of pebrine disease. However, these methods are not effective enough to completely eliminate the infection. Of the several therapeutic drugs, Benomyl, Nosematol, Bavistin and Thiophanate have been identified as anti-microsporidian agents to control *N. bombycis* infections (Alenkseenork, 1986; Chandra & Sahakundu, 1983). Although these fungicides have been proven to be experimentally effective in reducing the multiplication of spores, further studies clearly showed that these fungicides could not significantly eliminate transovarian transmission. *N. bombycis* is made to be inactive by hilite (Potassium dichloro isocyanurate) (Iwano & Ishihara, 1981). Baig *et al.* (1988b) studied the comparative efficacy of four disinfectants (*viz.,* hilite, sodium hypochlorite, bleaching powder and formalin) in four concentrations of 0.5%, 1.0%, 1.5% and 2% as surface sterilents against the spread of pebrine disease in

a colony of silkworms hatched from the surface contaminated laying and reported that all the tested concentrations were effective in preventing the spread of the disease and also successful in inactivating the spores of *N. bombycis* when exposed to 5, 10, 20 and 30 minutes, respectively. Kagawa (1980) studied the efficacy of formalin as a disinfectant against pebrine and reported an increased death rate of the spores with a raise in the concentration and temperature of formalin. Iwano and Ishihara (1981) tested nine chemical types as inhibitory agents against *N. bombycis*, with high degree of inhibitory effects on the spores.

However, the methods attempted to control pebrine disease by several authors have been found to yield limited success. Therefore, development of better and more reliable diagnostic methods to detect pebrine during seed production and silkworm rearing has always remained one of the most important and valid strategies to eliminate the disease from silkworm crops. Relatively recently, delayed mother moth test is recommended as a significant step in the area of pebrine disease diagnosis in microscopic test. In this method, the female moths are preserved alive at room temperature for a period of 3-4 days after oviposition and before subjecting for microscopic test. This allows improved sporulation of the pathogen to facilitate an easy and a more accurate detection of the disease (Samson, 2000). It has been reported that the multiplication rate of *N. bombycis* increases substantially with the age of moths and that the cephalothoracic region has the highest spore concentration, especially around the wing and wing muscles (Sasidharan *et al.,* 1994). Therefore, testing silk moths around 3-4 days after oviposition is a more effective way or method to detect pebrine with a much better accuracy. An improved testing method has also been recommended for better detection at egg stage. A sample of eggs was incubated at a moderately higher temperature of 32±1ºC for 48 h to enhance the sporulation of *N. bombycis.* Testing of such eggs will therefore enhance the chances of disease detection. On these lines and based on the principles of immunology, even the diagnostic techniques were also attempted in several countries, including India, for the detection of pathogen and spore identification, but with only limited success (Baig *et al.,* 1992).

N. bombycis and closely related spores were diagnosed using several techniques such as antibody-sentisized latex agglutination (Hayasaka & Ayuzawa, 1987), slide agglutination (Baig *et al.,* 1992; Li, 1985), ELISA procedures (Kawarabata & Hayasaka, 1987), fluorescent antibody (Sato *et al.,* 1981, 1982), serological (Grobov & Rodionova, 1985) and SPA coagglutination (Mei & Jin, 1998), etc. The development of the monoclonal antibody technique, which has very high specificity and stability, has played a great role in the studies of the classification and identification of specific microsporidians (e.g., Carlos *et al.,* 1996; Chen *et al.,* 1989). Meanwhile, Ke *et al.* (1990) raised monoclonal antibodies against *N. bombycis* spores and applied

them to identify pebrine and other closely related microsporidian spores infecting silkworms using the ELISA procedure. Shi and Jin (1997) reported that agglutination test using N5 McAb (hybridoma cell lines secreting monoclonal antibody) sensitized latex particle was a very practical technique for the diagnosis of the pebrine disease. Nonetheless, a simple dipstick immunoassay method tried later for the diagnosis of pebrine was also unsuccessful in the field. A simple negative staining procedure (Geethabai *et al.,* 1985) and an immunoperoxidase staining procedure (Han & Watanabe, 1987; Kawarabata & Hayasaka, 1987) were developed for the clarity during the examination of spores. Sironmani (1997) developed the Western blot method to identify the microsporidian infection and observed that immunological reaction with *N. bombycis* infected silkworm larvae and eggs showed the presence of 17-kDa polypeptide, which is specific to infection. The researcher further reported that 17-kDa polypeptide could be used as a virulent marker for the identification of microsporidian infection. DNA based probes have also been developed to identify *N. bombycis* (Malone & Mclvor, 1995).

Based on the amplification of rRNA gene fragments, several PCR methods are available for the diagnosis and species identification of insect microsporidia (Kawakami *et al*., 1995, 2001). The molecular techniques developed were found to have more sensitivity and specificity in the detection of the disease (Hatakeyama & Hayasaka, 2001). Nageswararao *et al*. (2004) studied the pathogenecity, mode of transmission, tissue specificity of infection and SSU-rRNA gene sequences for the microsporidian isolates from the silkworm, *Bombyx mori*. Using inter-simple sequence repeat PCR (ISSR-PCR) analysis, the genetic characterization and relationship between different microsporidia infecting mulberry silkworms have been reported (Nageswararao *et al*., 2005). The researchers further differentiated six different microsporidians through molecular DNA using ISSR-PCR and stated that the ISSR-PCR analysis might emerge as a powerful tool to detect, diagnose and identify microsporidians using inter simple sequence repeat PCR (ISSR-PCR) analysis, as it is difficult to study with microscope because of their extremely small size. A new technique based on the identification of intermediary stages has also been suggested for diagnosing pebrine (Santha *et al.,* 2001).

Although these tests are simple and sensitive, they still cannot create any impact on the pebrine disease diagnosis in the field, unless standard methods are evolved for their effective field applicability. To maintain the quality of silkworm eggs, several attempts have been made from time to time to improve the sampling procedure (Fujiwara, 1993; Kurisu, 1986; Kurisu *et al.,* 1985). Moreover, procedures have also been developed for the detection of pebrine spores in soil/dust, rearing and grainage houses, on mulberry leaves, eggshells/unhatched eggs, litter, etc. (Singh & Saratchandra, 2004). The sample size for the examination of faecal matter to

detect the presence of pebrine has also been described by Patil *et al.* (2001). As it is not possible to examine all the emerging moths in the commercial grainages, Fujiwara (1993) suggested a 20% sampling method and reported the probability of detection of the pebrine disease, as shown in Table 4.

Destruction of disease-causing microorganisms at various levels is a general method used in preventing and controlling the disease. Surface sterilization of disease free laying, maintenance of strict sanitation, hygienic rearing, frequent and careful examination of stock, disinfections of rearing rooms and appliances, removal of dead and infected larvae are to be strictly adopted to get rid of the disease. Meanwhile, exposing all the contaminated materials and equipments to direct sunlight, and disinfections with 2% formalin solution or 5% bleaching powder solution are the most effective and simple eradication methods for the disease. However, the pathogen killing action of the disinfectants is influenced by several factors such as temperature, humidity, concentration of disinfectants and duration of treatment (Kagawa, 1980). Recently, a new disinfectant chlorine dioxide (serichlor) is considered as an ideal disinfectant for all types of rearing/ grainage houses. In combination with slaked lime, it is 2.5 times stronger than chlorine and 2 times stronger than sodium-hypochloride. Furthermore, it is the least corrosive and also non-hazardous. When no single technique is sufficient enough to be used in checking the disease in field, it becomes obligatory to choose a multi-pronged approach. Unfortunately, the technique can only assist in detecting the

TABLE 4
Probability of detection of pebrine in 20% sampling method (Index)

No. of egg cards (20 layings on each card)	Population (No. of layings)	Pebrine	Samples	Probability	
				Non Detectable	Detectable
20	400	2	80	0.6400	0.3600
30	600	3	120	0.5120	0.4880
40	800	4	160	0.4096	0.5904
50	1000	5	200	0.3277	0.6723
60	1200	6	240	0.2621	0.7379
80	1600	8	320	0.1678	0.8322
100	2000	10	400	0.1074	0.8926
150	3000	15	600	0.0352	0.9648
200	4000	20	800	0.0115	0.9885
250	5000	25	1000	0.0380	0.9620
300	6000	30	1200	0.0012	0.9988
500	10000	50	2000	0.0000	1 .0000

Rate of pebrine infection = 0.05% in female moths

(Source: Fujiwara, 1993)

disease and the only way out is to destroy the diseased silkworm crops which cause loss, apart from making efforts to prevent the said disease at all levels.

A burning problem in the field of microsporidiosis is the increasing number of different microsporidians that are being encountered in silkworm crops (Fujiwara, 1980, 1993). These microsporidians have been shown to exhibit varying degrees of virulence and many of them have demonstrated low multiplication rate in silkworm although they are infective and pathogenic. Some of them have not shown vertical transmission in the host. As of today, however, there has been no specific testing procedure to discriminate these microsporidians in the field to take appropriate action, while preparing disease free silkworm seed. If pebrine is to be controlled effectively, a system has to be evolved, where either a seed cocoon grower or a seed producer is not put in hardship due to the reoccurrence of the disease.

Future Research Strategies

Application of the molecular techniques for diagnosis, species differentiation, identification of intermediary stages of development, multiprimer PCR techniques will lead to enormously increased knowledge of the microsporidians infecting silkworms (*Bombyx mori*) in the near future. In addition, there is also a need to develop better, rapid, systematic and feasible techniques for early detection of the disease, to evolve pebrine resistant region and season specific breeds/ hybrids of silkworm for commercial use, and to identify the potential target organs of both the parasites and host for control through chemical agents, apart from elaborating serological and epidemiological studies in natural epizootics involving biology, host parasite interactions, taxonomy, etc. and developing effective and efficient model(s) for forecasting of the disease outbreaks.

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Short Communications

An Urgent Need for Milky Stork Study in Malaysia

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ABSTRACT

Milky Stork (*Mycteria cinerea*) once had a scattered distribution in the West coast of Peninsular Malaysia. The species later underwent a constant decline and it now has less than 10 individuals recorded in Matang Mangrove Forest. Among the problems are threats from the pollution of hazardous chemicals, habitat destruction, poaching by humans, high rates of predation and disturbance, and the lack of mature trees for nesting. Thus, identification of suitable habitats for Milky Stork routine activity is important and Matang Mangrove Forest has provided such an opportunity for the Milky Stork Conservation Programme. In addition, there is also a need for integrated studies from various disciplines to conserve the remaining Milky Stork in Malaysia from extinction.

Keywords: Malaysia, Matang Mangrove Forest, Milky Stork, population decline, conservation

INTRODUCTION

The Milky Stork (*Mycteria cineria*) can be found throughout Southeast Asia, with a status of rare to local residents in Peninsular Malaysia (Robson 2002). The birds once had a scattered distribution in the Peninsular, ranging from the coasts of Kedah, Perak, Selangor, Malacca and Penang (Robinson & Chasen, 1936; Gibson-Hill, 1949). This species was also reported to have existed at least in East Malaysia with a rare status and much localised (Marioka & Yang, 1990). The population of Milky Stork, however, has undergone a constant decline since 1980s (Li *et al*., 2006), suggesting the reduced number of breeding successes, increased predation rate and illegal hunting in the wild. The decreasing pattern in the wild population greatly affected two species of storks in Malaysia; namely, Milky Stork and Painted Stork, whereby both are listed as vulnerable and near threatened species,

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respectively (IUCN, 2010). The current population of wild Milky Storks in Malaysia is restricted to Matang Mangrove Forest Reserve near Kuala Gula, Perak. Mangrove forest is the most suitable habitat for Milky Storks. Since they are specialised in the mangrove forest, there are many biological and ecological aspects related to intertidal environment, mangrove ecosystem and birds' life history that need to be studied.

This paper highlights an important issue in the conservation of Milky Stork in Malaysia. The discussion is based on the limited literature review available and the researchers' personal involvement in research related to the breeding of Milky Stork programme in captivity, releasing them into the wild environment and other ecological aspects of the coastal environment, mangroves and habitat quality of coastal birds.

As highlighted by many authors, the pollution of hazardous chemicals is among the important issues in the conservation of wildlife, such as coastal birds, apart from the ecological (such as habitat changes, presence of natural predators, etc.) and human factors (such as forestry and fishing activities, and illegal hunting). Increased shipping activities and rapid development in the coastal areas of Peninsular Malaysia have increased the loads of pollutant inputs, such as Tributyltin (TBT), heavy metals, pesticides and nutrients (Ismail *et al*., 2003; Monirith *et al*., 2003; Sudaryanto *et al*., 2004; Agusa *et al*., 2005) into the coastal environment. The heavy metal contamination in the Malaysian coastal environment is also well described (Ismail *et al*., 1991, 1993; Ismail *et al*., 1995; Shahrizad *et al*., 2003), along with other pollutants such as plastic pellets (Ismail & Riak, 2003; Ismail *et al*., 2009) that possess threats to the water birds. Meanwhile, bioaccumulation of the pollutants in sediments (Ismail *et al*., 1993), fish (Agusa *et al*., 2005) and prawns (Ismail *et al*., 1995) also threaten Milky Stork as they biomagnified in the food webs system. As most of the Milky Stork foraging areas are along the coastal line, such species are in eminent danger of being contaminated by these pollutants. The effects of contamination on water birds (De Luca-Abbot *et al*., 2004; Horai *et al*., 2006; Ayas, 2007; Kim & Koo, 2007) include among others, thinning of eggshells, premature hatching, and deformities in their young. Such impacts are detrimental to the water birds population, particularly the Milky Stork species. Thus, analysis of those chemicals in the birds' habitat is important for the purpose of conservation since the data may reflect both the quality and health of their habitat.

Verheugt (1987) highlighted habitat destruction, timber exploitation and poaching by humans as the main threats responsible for the decline of Milky Storks in the wild in the 1980s. Ecologically, the lack of mature forest trees for nesting, high rate of predation and habitat disturbance are some other reasons behind the declining population of Milky Stork (Li *et al*., 2006). Even though some trials have been conducted for rehabilitation and conservation of Milky Storks, such as the ones in Kuala Selangor in

1998 and Kuala Gula in 2006, the projects were not very successful at least at the time when this article was written. There are probably many reasons why the projects have failed and urgent attention is therefore needed.

Among the important reasons why the initiatives are not thriving are the lack of information on the ecology and behaviour of Milky Stork in the wild, their ability to adapt in the wild environment, and public support, as well as conservation awareness and understanding. Therefore, immediate actions taken to gather information relevant to ecological, biological and sociological factors, along with studies on Milky Storks in Malaysia and the neighbouring countries (Indonesia, Vietnam, Cambodia and Thailand) and public education, are important and urgently needed.

Recently, the government of Malaysia, through the Department of Wildlife and National Parks (PERHILITAN) and international agencies, has shown great care and awareness towards the conservation of Milky Storks in Malaysia. Pulau Kelumpang is an important site associated with Milky Storks in Malaysia. The area is the last known place where wild Milky Storks sightings have been recorded. For example, Siti Hawa (1989) reported that 130-150 Milky Stork individuals in Pulau Kelumpang between 1984 and 1989. In addition, a number of Milky Stork nests were also recorded at the time of that survey. Rahmah *et al*. (2007) also reported that nesting attempts were observed in the area, but these were to no avail. Nonetheless, the failure of the birds' nesting attempts was not well described. A review of the Milky Stork status by Li *et al*. (2006) highlighted that there were less than 10 wild individuals Milky Stork observed at Matang Forest Reserve, particularly in Pulau Kalumpang. Moreover, the information gathered showed that the population had undergone a decline of more than 90% over the last 20 years. If this report is true, the population status of the Milky Storks in this country is extremely critical. In a recent study between August and December 2009, less than five individuals were observed in the wild around Pulau Kalumpang and Pulau Terong (Ismail *et al*., 2010).

Malaysia is very lucky because there are still a large number of Milky Storks in captivity. The status of the captive breeding programme in Zoo Negara has been summarized by Ismail *et al*. (2011). Up to 2005, about one hundred Milky Storks are living and breeding in captivity at Zoo Negara in Hulu Kelang, Selangor. They are a very important colony and have a great potential in the conservation programme. Malaysian Zoological Society, Zoo Negara, Wildlife Department Malaysia and Universiti Putra Malaysia are trying very hard to conserve and increase the number of Milky Storks in the wild, particularly in Kuala Gula. Nonetheless, the breeding programme in captivity may also face a few problems. Among the problems are incorrect feeding method to chicks, collapses of nest trees, as well as weakening of pair bond through egg manipulation and storm damages (Yaacob, 1994). However,

all these problems have been gradually overcome by the management of Zoo Negara who has been able to increase the birds' population since 1987.

Meanwhile, the Wildlife Department of Malaysia and Universiti Putra Malaysia conducted a brief study on the adaptability of the released Milky Storks in to their natural environment at the mangrove forest in Kuala Gula, Perak, which is located about 300 kilometres north of Kuala Lumpur. This brief study has suggested that a few modifications and adaptations be done in order to ensure the survival of the birds released into the wild. These include improvement of the cage area and the surrounding environment, modification of the feeding technique used for captive birds and some ecological aspects that need to be looked into (Ismail *et al*., 2010). Based on the current issues related to the population of Milky Storks, some previous studies conducted and government's concern, there is an urgent need to study all the aspects of the Milky Stork biology, ecology and habitat, both in the wild and in captivity. Among other, the integrated approaches involving biologists, ecologists, foresters, educationists and sociologists are urgently needed before the Milky Stork species becomes totally extinct in its own environment.

In order to establish an integrated study on Milky Storks, a potential site is needed. Matang Forest Reserve is one of the ideal locations to study and conserve Malaysian Milky Storks. This forest reserve is currently managed by the Forestry Department and the trees in each compartment are felled in a 20-30 years cycle. If this condition is strictly followed, the trees would have matured enough to reach the height required by Milky Storks to build their nests. Furthermore, the area is 53 kilometres in length and 13 kilometres in width, and it is located near Kuala Gula Bird Sanctuary, which is well-managed and protected; this condition is enough to support a small population of the wild Milky Storks and protect them from extinction. As they are specialised in mangrove forest, they can therefore be a key species for the mangrove ecosystem. Their existence in the mangrove ecosystem may reflect the ecosystem stability and balance in several aspects, including the stability of the mangrove trees, as well as the tropic levels and the ecology of the mangrove forest. An integrated research on the ecology and biology of birds, the ecology of mangrove forests, intertidal mudflat ecosystem, fisheries, as well as benthos ecology and pollution status in this specific location are needed to ensure that the conservation of Milky Storks in Malaysia is promising. Therefore, an urgent action from various disciplines of science, active participation of the local and foreign scientists and a special budget are all needed in order to support the conservation of Milky Storks in Malaysia. Considering the dire situation of the wild Milky Stork population in Malaysia, a permanent and suitable site is very important for the purpose of their conservation. In addition, systematic actions must be taken to rehabilitate the species in the identified

area to ensure the survivorship of the local Milky Storks.

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Distributions of Cu and Zn in the Shell Lipped Part Periostracum and Soft Tissues of *Perna viridis*: The potential of Periostracum as a Biomonitoring Material for Cu Contamination

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ABSTRACT

Periostracum is the outer shell layer composes mainly of organic materials. In the present study, the green-lipped mussel *Perna viridis* was used to investigate the distributions of Cu and Zn in the periostracum and soft tissues of the *P. viridis* which were sampled from 17 geographical sites [23 populations] along the coastal waters of Peninsular Malaysia. The concentrations of Cu in the periostracum and the soft tissues of *P. viridis* were 7.41-42.63 μg/g dry weight and 3.49-31.1 μg/g dry weight, respectively. Meanwhile, the concentrations of Zn in the periostracum and soft tissues of *P. viridis* were 4.90-39.79 μg/g dry weight and 65.75-144.9 μg/g dry weight, respectively. The ratios of the metals in periostracum to soft tissues were 0.73-3.99 μg/g for Cu and 0.05-0.36 μg/g for Zn. These ratios indicated that the concentrations of Cu in the periostracum were generally greater than those in the soft tissues while the concentrations of Zn were generally higher in the soft tissues than those in the periostracum. The higher Cu levels in the soft tissues compared to that in the periostracum (Fig. 2) and the relatively close relationships of Cu between periostracum and sediment indicated that the periostracum was a good biomonitoring material for Cu, but periostracum was not a good biomonitoring material for Zn because it did not reflect the environmental contamination as reflected in the low correlation between the periostracum and sediment.

Keywords: Periostracum, *Perna viridis*, biomonitoring material, Cu and Zn

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INTRODUCTION

Soft tissues of marine bivalves have been frequently used in the biomonitoring studies of heavy metal pollutions in coastal waters. Several researchers used molluscs' shells as biomonitors of heavy metal pollution

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and their studies have been documented (Bertine & Goldberg, 1972; Koide *et al*., 1982; Bourgoin & Risk, 1987; Foster & Chacko, 1995; Ravera *et al*., 2001; Szefer *et al*., 2002; Brown *et al*., 2005, Cravo *et al*., 2002, 2005; Yap *et al*., 2003a, 2004). For example, Cravo *et al*. (2002) found that the shell of limpets *Patella aspera* is a tissue for potential use in environmental trace metal monitoring based on the marked and significantly higher levels of Fe and Mn in the contaminated site compared to the reference site. Brown *et al*. (2005) utilized the freshwater mussel shells to assess mercury (Hg) contamination in the North Fork Holston River. Foster and Cravo (2003) found *Nerita albicilla* as having the greatest potential as a biomonitoring tissue for trace metals, whereas Gillikin *et al*. (2005) assessed the use of clam shells (*Mercenaria mercenaria*) as a proxy of lead pollution.

The rationales of using bivalve shells in the study of heavy metal pollution were made based on several positive arguments and the characteristics of the shell formation. Each year, a mussel produces an incremental layer of its shell which is composed mainly of calcium carbonate and a small fraction of organic substance (Lindh *et al*., 1987). Many other elements are simultaneously deposited in these annual layers and are assumed to be essentially immobile after biodeposition into the crystalline lattices of the shell structure (Yap *et al*., 2003a). In the process of shell secretion, it is the mantle epithelium which secretes the extrapallial fluid. The fluid contains the components for biomineralization (Ca^{2+}, HCO^{3-}, organic molecules) and may also contain heavy metals if these are present in the outer medium (Watson *et al*., 1995). Any trace metals actively incorporated within the shell matrix during shell growth must have been assimilated by the organism (Wilbur & Saleuddin, 1983).

The shell of most bivalves is generally composed of an outer organic layer, the periostracum and two calcareous layers, namely the prismatic and nacreous (Gordon & Carriker, 1980). The periostracum layer is entirely organic and produced at the edge of mantle. The periostracum consists of proteins that have been sclerotized by quinone tanning to give the characteristic of horny texture (Gordon & Carriker, 1980). Several investigators of the periostracum in different species of bivalves have shown that it is composed of quinine tanned protein, mucopolysaccharides and lipids, and that some consist of several layers with different structure and staining properties. In addition, it is also thought that the two main functions of the periostracum are; firstly, to provide a waterproof covering for the shell, protecting it from acid dissolution and, secondly, to provide a substratum upon which calcium carbonate crystals can be deposited initially at the edge of the shell (Nakahara & Bevelander, 1971).

To our knowledge, little is known about the concentrations of Cu and Zn in the periostracum of green-lipped mussel *Perna viridis* in the literature. Therefore, the objective of this study was to determine the distributions and concentrations of Cu

and Zn between the periostracum and soft tissues of the *P. viridis* which had been collected from 17 geographical sampling sites in Peninsular Malaysia, including those collected from different environmental backgrounds.

MATERIALS AND METHODS

Mussels *P. viridis* were collected from 17 geographical sites (23 populations) along the coastal waters of Peninsular Malaysia (Fig.1). Surface sediments (0-10 cm) were also collected from 9 sampling sites (Table

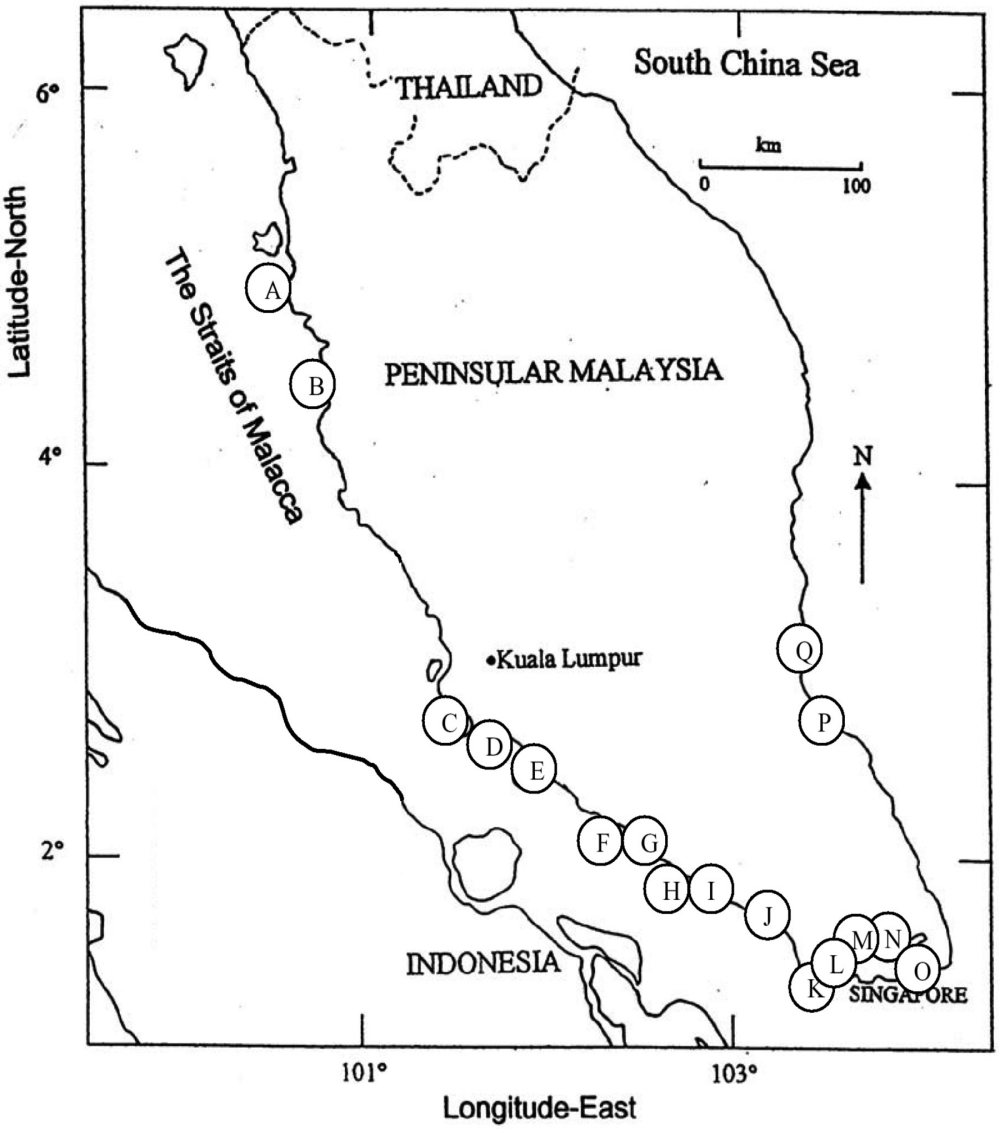

Fig.1: The sampling sites of the green-lipped mussel *Perna viridis* from the coastal waters of Peninsular Malaysia. Names of the sampling sites are represented by the alphabets as shown in Table 1

TABLE 1
Sampling dates and shell lengths (mean ± SE) of *Perna viridis* analyzed and descriptions of the sampling sites in the coastal waters of Peninsular Malaysia. Shell lengths are in mm. About 15-20 individuals were analysed in each sampling site

	Sampling sites	Sampling dates	Shell lengths	Descriptions of sampling site
A	*Pulau Aman (Penang)	11 Sep1999	91.50 ± 2.0	A fish aquacultural area
B	Bagan Tiang-1 (Perak)	01 Apr 2002	135.0 ± 6.0	An aquacultural area
	Bagan Tiang-2 (Perak)	20 Apr 2005	86.5 ± 3.0	An aquacultural area
C	*Bagan Lalang (Selangor)	08 Jun 1998	91.2 ± 4.5	A recreational and agricultural areas
D	*Lukut (Negeri Sembilan)	08 Aug 1998	93.9 ± 3.1	An aquacultural area
E	*Pasir Panjang (Negeri Sembilan)	22 Sep 1998	88.6 ± 2.6	A mussel aquacultural area
F	*Kuala Linggi (Negeri Sembilan)	21 Nov 2000	80.0 ± 1.2	A fish and mussel aquacultural area
G	Merlimau-1 (Malacca)	19 Apr 2002	68.55 ± 2.5	A mussel aquacultural area
	Merlimau-2 (Malacca)	09 Apr 2004	82.30 ± 3.8	A mussel aquacultural area
H	Telok Emas (Malacca)	09 Apr 2004	84.61 ± 4.8	A mussel aquacultural site
I	*Sebatu-1 (Malacca)	12 Aug 2000	85.4 ± 4.5	A mussel aquacultural area
	Sebatu-2 (Malacca)	19 Feb 2002	63.04 ± 3.8	A mussel aquacultural area
J	Minyak Beku (Johore)	18 Jan 2005	76.0 ± 1.0	Wild mussels found a recreational site.
K	Kukup (Johore)	18 Jan 2005	83.0 ± 3.0	Wild mussels found at a fish agricultural floating house and near a jetty.
L	*Tg. Kupang (Johore)	19 Jan 2000	83.6 ± 3.0	A port and an aquacultural area.
M	*Pantai Lido-1 (Johore)	23 Sep 1998	59.4 ± 2.7	Urban and restaurant areas.
	Pantai Lido-2 (Johore)	17 Apr 2002	89.08 ± 5.0	Urban and restaurant areas.
	Pantai Lido-3 (Johore)	19 Jan 2005	103.0 ± 6.8	Urban and restaurant areas.
N	*Kg. Pasir Puteh-1 (Johore)	19 Jan 2000	61.10 ± 2.1	Industrial and mooring activities and urban areas
	Kg. Pasir Puteh-2 (Johore)	17 Apr 2002	92.84 ± 3.3	Industrial and mooring activities and urban areas
O	Kuala Belungkor (Johore)	18 Apr 2002	64.94 ± 1.2	Pristine area and a fish aquacultural area.
P	Kuala Pontian (Pahang)	08 Apr 2004	70.11 ± 1.5	A mussel aquacultural site.
Q	Nenasi (Pahang)	08 Apr 2004	76.11 ± 2.3	A light house nearshore; pristine water.

Note:* indicated where the sediments were also collected in the mussel habitats.

TABLE 2
The Cu concentrations (mean μg/g dry weight) in periostracum (perios) and the total soft tissues (ST) of *Perna viridis* (N= 3)

	Sampling sites	Perios	ST	$\frac{Cu_{perios}}{Cu_{ST}}$
A	Pulau Aman	22.99	10.80	2.13
B	Bagan Tiang-1	13.24	14.14	0.94
	Bagan Tiang-2	7.41	9.86	0.75
C	Bagan Lalang	26.09	8.20	3.18
D	Lukut	24.50	10.22	2.40
E	Pasir Panjang	17.76	10.87	1.63
F	Kuala Linggi	24.03	9.14	2.63
G	Merlimau-1	15.63	11.07	1.41
	Merlimau-2	18.95	8.94	2.12
H	Telok Emas	12.97	3.49	3.72
I	Sebatu-1	14.99	11.16	1.34
	Sebatu-2	17.97	15.72	1.14
J	Minyak Beku	8.63	10.21	0.85
K	Kukup	13.22	11.68	1.13
L	Tg.Kupang	15.39	6.31	2.44
M	Pantai Lido-1	20.50	9.39	2.18
	Pantai Lido-2	18.88	14.27	1.32
	Pantai Lido-3	11.47	15.71	0.73
N	Kg.Pasir Puteh-1	29.49	20.10	1.47
	Kg.Pasir Puteh-2	42.63	31.09	1.37
O	Kuala Belungkor	16.67	7.96	2.09
P	Kuala Pontian	20.28	14.00	1.45
Q	Nenasi	18.53	4.64	3.99

1). All the samples were stored at -10°C until metal analysis. At the laboratory, the soft tissues were carefully separated from the shell and the byssus was discarded. The periostracums were pooled and triplicates were analyzed; 15-20 individuals of the total soft tissues of mussel *P. viridis* were analyzed in each sampling site (*see* Table 1).

After rinsing with double distilled water and 0.5% HCl, they were dried for 72 hours at 105°C to constant weights (Mo & Neilson, 1994). In order to separate the outermost layers (periostracum layers) of the shells, they were cooled at room temperature after heating at 105°C. While the shells were cooling, most of the outer layers cracked and fell off (Puente *et al*., 1996). The periostracum layers were weighed with an accuracy of 0.1 mg before acid digestion. The periostracum and soft tissues were digested in concentrated HNO_3 (AnalaR grade; BDH 69%).

The sediment samples were also dried at 105°C to constant weights. The dried sediment samples were crushed using a mortar and pestle and then sieved through a 63 μm aperture stainless steel sieve before they were shaken vigorously to produce homogeneity. For the analyses of the total Cu and Zn concentrations in the sediment samples, three replicates were analyzed using the direct aqua-regia method. About 1g of each dried sample was digested in a combination of concentrated HNO3 (AnalaR grade; BDH 69%) and HClO4 (AnalaR grade; BDH 60%) in the ratio of 4:1 (Yap *et al*., 2002a).

The periostracum, total soft tissues and sediment samples were put into a hot-block digester first at low temperature (40°C) for 1 hour and then were fully digested at 140°C for at least 3 hours. The digested samples were then diluted to a certain volume (40 ml) with double distilled water.

TABLE 3
The Zn concentrations (mean μg/g dry weight) in the periostracum (perios) and total soft tissues (ST) of *Perna viridis* (N= 3)

	Sampling sites	Perios	ST	$\frac{Zn_{perios}}{Zn_{ST}}$
A	Pulau Aman	19.92	109.6	0.18
B	Bagan Tiang-1	7.67	65.75	0.12
	Bagan Tiang-2	4.90	101.8	0.05
C	Bagan Lalang	6.25	96.36	0.06
D	Lukut	11.51	69.40	0.17
E	Pasir Panjang	8.15	98.92	0.08
F	Kuala Linggi	13.09	101.1	0.13
G	Merlimau-1	11.34	88.64	0.13
	Merlimau-2	39.79	111.8	0.36
H	Telok Emas	6.84	102.1	0.07
I	Sebatu-1	6.80	75.14	0.09
	Sebatu-2	14.59	105.4	0.14
J	Minyak Beku	11.58	139.4	0.08
K	Kukup	15.89	140.6	0.11
L	Tg.Kupang	13.82	88.58	0.16
M	Pantai Lido-1	13.23	80.49	0.16
	Pantai Lido-2	12.60	105.5	0.12
	Pantai Lido-3	20.95	135.8	0.15
N	Kg.Pasir Puteh-1	15.70	128.9	0.12
	Kg.Pasir Puteh-2	9.60	69.89	0.14
O	Kuala Belungkor	6.81	86.26	0.08
P	Kuala Pontian	24.00	140.3	0.17
Q	Nenasi	8.03	96.50	0.08

TABLE 4
Overall mean concentrations, minimum and maximum values of Cu and Zn in the periostracum (Perios) and total soft tissues (ST) and their ratios (Perios/ST) of *Perna viridis*. N= 23 populations

		Minimum	Maximum	Mean	SE
Zn	Perios	4.90	39.79	13.18	1.60
	ST	65.75	140.63	101.67	4.80
	Perios/ST	0.05	0.36	0.13	0.01
Cu	Perios	7.41	42.63	18.79	1.57
	ST	3.49	31.09	11.69	1.18
	Perios/ST	0.73	3.99	1.84	0.19

After filtration, the prepared samples were determined for Cu and Zn by using a flame atomic absorption spectrophotometer (AAS) Perkin-Elmer Model 4100. The data were presented in μg/g dry weight basis. To avoid possible contamination, all glassware and equipment used were acid-washed. In addition, our analytical procedures were checked with Certified Reference Materials for Dogfish liver (DOLT-3) and the recoveries were being satisfactory (Cu: certified = 31.20 μg/g dw; measured =32.0 μg/g dw and Zn: certified = 86.6 μg/g dw; measured = 100.3 μg/g dw).

RESULTS

From Tables 2 and 4, the concentrations of Cu in the periostracum and soft tissues of *P. viridis* were 7.41-42.63 μg/g dry weight and 3.49-31.1 μg/g dry weight, respectively. The ratios of Cu periostracum/soft tissues are between 0.73 and 3.99. Except for four populations (out of 23 populations), the Cu levels were higher in the periostracum than in those in the soft tissues, as indicated in Fig.2. The highest Cu concentration in the periostracum was found at Kg. Pasir Puteh-2 collected in 2002 (42.6 μg/g dry weight), followed by Kg. Pasir Puteh-1 (29.5 μg/g dry weight) which was sampled in 2000. These results were supported by the higher Cu levels in the soft tissues of the similar sampling sites and the high Cu levels in the sediments collected from Kg. Pasir Puteh (Yap *et al*., 2002a). Therefore, this good relationship had provided a basis for the reason why the periostracum could be used as a potential biomonitoring material for Cu pollution.

From Tables 3 and 4, the concentrations of Zn in the periostracum and soft tissues of *P. viridis* were 4.90-39.79 μg/g dry weight and 65.75-144.9 μg/g dry weight, respectively. The ratios of Zn periostracum/ soft tissues are between 0.05 and 0.36. All the 23 populations showed higher levels of Zn in the soft tissues than in the periostracum, as indicated in Fig.2. Meanwhile, the highest Zn concentration in the periostracum was found at Merlimau-2 (39.8 μg/g dry weight), whereas the lowest one was found at Bagan Tiang (4.90 μg/g dry weight). However, these results are not supported by the Zn levels in the soft tissues of the similar sampling site.

The relationships of Cu and Zn between the periostracum or total soft tissues and the sediments are presented in Figure 3. It was found that the soft tissue-Cu is highly correlated (R= 0.83) with sediment-Cu while the soft tissue-Zn is also correlated well (R= 0.73) with sediment-Zn. The lower correlation in Zn as compared to than Cu indicated that Zn is most likely to be partially regulated (Yap *et al*., 2002b). However, the soft tissues of *P. viridis* are usually used for the biomonitoring of Cu and Zn in the tropical coastal waters. As shown in Figure 3 again, periostracum-Cu is found to be correlated (R= 0.61) with sediment-Cu, while periostracum-Zn is not well correlated (R= 0.33) with sediment-Zn. The insignificant correlation between periostracum-Zn vs. sediment-Zn could be due to lower Zn levels in the periostracum than those in the soft tissues (see Figure 2). The higher Cu levels in the soft tissues than in the periostracum (Figure 2) and the relatively close relationship of Cu between periostracum and sediment indicated that the periostracum was a good biomonitoring material for Cu, compared to periostracum which was not a good biomonitoring material for Zn because it did not reflect the environmental Zn contamination, as indicated by the low correlation between the periostracum and sediment.

DISCUSSION

The distribution or partitioning of Cu and Zn in the periostracum and the soft tissues of *P. viridis* indicated three important points from the biomonitoring points of view. First, the differences in the accumulations of Cu and Zn in the hard and soft tissues which could be due to the differences of the binding affinities to sites between the periostracum, which is mainly composed of organic materials, whereby the soft tissues are

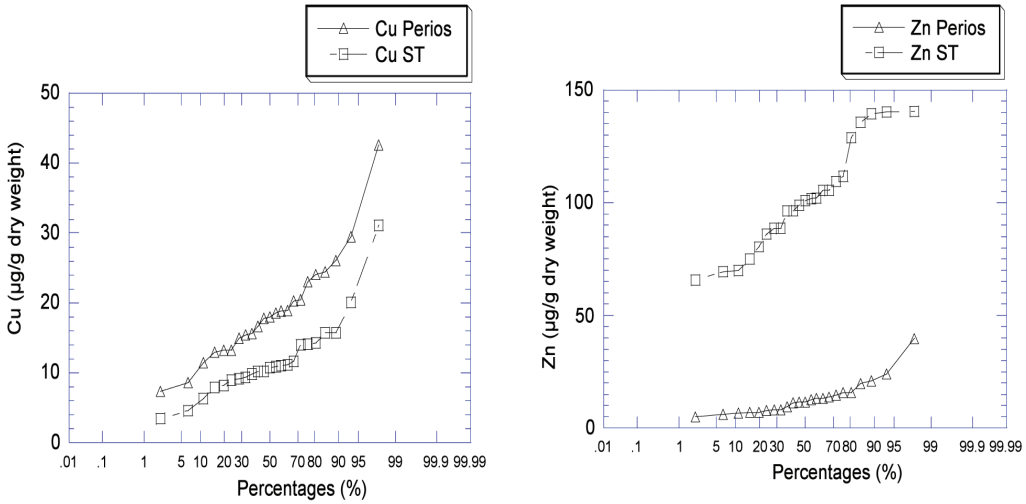

Fig.2: The probability of concentrations of Cu and Zn in the periostracum (Perios) and total soft tissues (ST) of *Perna viridis*. N= 23 populations

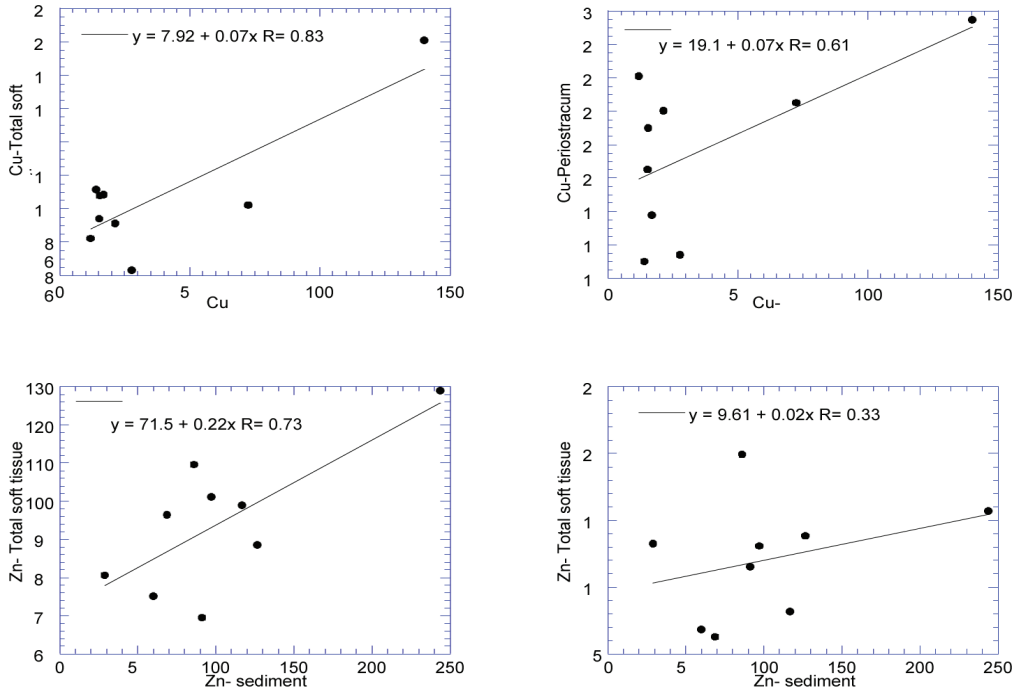

Fig.3: The relationships of Cu and Zn between the periostracum or total soft tissues and sediments. N= 9 populations

mainly composed of organic and inorganic materials. Second, the higher Cu levels in the periostracum than those in the soft tissues and the relatively close relationship of Cu between periostracum and sediment are the findings which support the use of periotracum as a potential biomonitoring material of Cu. In addition, the highest Cu concentration in the periostracum was found in the known Cu-contaminated site at Kg. Pasir Puteh (Yap *et al*., 2002a, 2003b). Since the periostracum is mainly composed of the green lipped part of the outer shell layer, the higher Cu levels in this shell layer is expected since the Cu concentration is responsible for the green colour of the lipped shell part (Yap *et al*., 2003a).

From the literature, the binding of Cu and Zn in the soft tissues of marine mussels has been shown to be due to the metallothionein synthesis for detoxification (Yap *et al*., 2006). However, the way in which the metals are incorporated into the shells of molluscs is due to the substitution of the calcium ion in the crystalline phase of the shell or associated with the organic matrix (Watson *et al.,* 1995). Moreover, the mineralogy and chemistry of the shell's material secreted by organisms can vary with the environment of growth (Dodd, 1963). Al-Dabbas *et al.* (1984) suggested that a shell's composition is sufficiently sensitive to environmental variation that the sub-environments can be distinguished within

a limited water system. This suggestion has further strengthened the hypothesis that the periostracum could be a potential biomonitoring material for Cu.

Although it has been reported that the periostracum shell layer is mainly composed of organic materials (Kennedy *et al*., 1969), the reason for the higher levels of Cu in the periostracum than those in the soft tissues is interesting from the biochemical point of view. Thus, 'how do the Cu levels bind to the organic materials of the shells?' should prompt further studies. However, the periostracum layer covers less than 5% of the total shell weight and therefore, a mussel shell mainly consists the two calcareous inorganic layers. This could be the reason why many researchers did not focus on the outer shell layer of the periostracum but instead examine on the inner or total shells of the mollusks.

The periostracum is a potential biomonitoring material for Cu because it has some advantages as a biomonitoring material. First, the shells themselves can also accumulate Cu to a considerable extent (Sturesson, 1976) and are higher than the soft tissues. Second, the comparison of metals between the newer shells and the material from collections or fossil shell, can also be made. Bourgoin and Risk (1987) studied the recent and fossil shells of *Mya truncata* (8200 a BP) and their surrounding sediments which were collected from three sites near Pangnirtung, Northwest Territories, in the eastern Canadian Arctic. The researchers found that the Pb levels in the fossil shells were approximately five times lower than those detected in the modern shells. Hence, the results of this study suggested that the determination of pollutant levels in the shells of bivalves might be an important and underutilized tool for environmental assessment.

Another advantage of using periostracum as a biomonitoring material is that it is easier to handle and to store; in fact, there is no need to preserve the shell materials under low temperature like the soft tissues. Furthermore, the problem of whether or not to depurate the animals before analyses is avoided (Koide *et al*., 1982). Brown *et al*. (2005) also proposed that the shell-based strategies based on freshwater mussels do not require sampling live specimens and might augment more standard strategies applied to environmental monitoring.

Thus, it is essential to choose a shell structural component that has not been exposed to either particulate or dissolved metals in the water column (Bourgoin, 1990) because these metals will also be incorporated by adsorption and cannot be distinguished from those metals which are truly assimilated (Phillips, 1980). The arguments and weaknesses of using the periostracum layer for the biomonitoring of trace metals are as follows:

1. The layer is exposed and therefore it is not the best bioavailability biomonitoring material;
2. It is the newly developed shell layer and therefore it does not provide an indicator of long-term exposure to heavy metal pollution in the coastal waters.

However, based on the findings of the

present study, the use of the periostracum as a good biomonitoring material of Cu is proposed because of three positive arguments listed below:

1. First, since it is exposed to environmental seawater, it is therefore a partially good indicator of environmental Cu in the seawater adsorbed onto the periostracum surfaces.
2. Second, it is a newly developed shell layer from the mantle edge of the mussels and therefore, the metals found in this layer contain biologically deposited Cu in the recent time.
3. Third, the relatively close relationship of Cu between periostracum and sediment positively supported that the periostracum of *P. viridis* was a good biomonitoring material for Cu since the Cu levels in the periostracum was found to be generally higher than those in the soft tissues. Therefore, the Cu levels of the periostracum could provide an index of Cu bioavailability, apart from reflecting the Cu contamination of the sampling site.

CONCLUSION

The higher Cu levels in the soft tissues compared to those in the periostracum (as indicated by the ratios of periostracum to soft tissues) and the relatively close relationship of Cu between the periostracum and sediment indicated that the periostracum was a good biomonitoring material for Cu but not for Zn. However, it is still unknown from the molecular point of view (DNA level) for reasons why the higher Cu levels have been found in the periostracum compared to the soft tissues, and on the other hand, a reverse pattern was found for Zn. All these should prompt more future studies on the use of periostracum and the inner shell layers of mussels as biomonitoring materials of heavy metal pollutions in coastal waters.

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Distribution of Heavy Metal Concentrations in the Different Soft and Hard Tissues of Tropical Mud-Flat Snail *Telescopium telescopium* (Family: Potamididae) Collected From Sepang Besar River

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ABSTRACT

The concentrations of Cd, Cu, Pb, Fe, Ni and Zn were determined in the different parts of the soft tissues (foot, cephalic tentacle, mantle, muscle, gill, digestive caecum and remaining soft tissues) and three parts of hard tissues or shells (anterior shell, middle shell and posterior shell) of the mud-flat snail *Telescopium telescopium* collected from Sepang Besar River. From the cluster analysis, the dendrogram shows that the three parts of the shells are clustered separately from the different parts of the soft tissues, indicating different mechanisms and strategies of metal accumulation and regulation of heavy metals in the shells from the different soft tissues. Among the different soft tissues, the dendrogram also shows that the digestive caecum is clustered differently from other soft tissues, indicating that this organ is distinctly high in metal accumulation and this may probably suggest a different route of metal sequestration from the rest of the soft tissues. The metal distribution found in the different soft tissues of *T. telescopium* is an important knowledge in establishing this mud-flat gastropod as a promising biomonitor of metal contamination and bioavailability for the intertidal area of Peninsular Malaysia.

Keywords: *Telescopium telescopium*, metal distribution, different tissues

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INTRODUCTION

It has been widely reported in the literature that gastropods accumulate metals in their tissues in proportion to the degree of environmental contamination and that they can be used as biomonitors of marine metallic pollution (Goldberg *et al*., 1978). The usefulness of molluscs, as sentinel

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organisms in metal biomonitoring studies, is widely recognized (see Rainbow, 1990, 1993; Langston & Spence, 1995; Brown & Depledge, 1998). Snails are good models for examining the effects of pollution on populations because they are in contact with polluted bottom sediments and have short generation time (Lefcort *et al*., 2004). Snails are also known to alter their locations in order to thermoregulate with great accuracy (Lefcort & Bayne, 1991). They are appropriate to be use as biomonitors in situ because they are sedentary, abundant, of relative longevity, large, as well as easily collected and weighed (Hartley & Johnston, 1983).

Most of the biomonitoring studies using gastropods have been directed either to the total soft tissues (e.g. Ismail & Safahieh, 2004) or to the shells, but very few have concurrently addressed trace metal concentrations in the different parts of both the soft and hard tissues. In general, the accumulation and storage of trace metals (e.g. Cd, Cu and Zn) in common biomonitors such as gastropods are strongly associated with the level and metal binding capacity of metallothioneins in their tissues (Roesijadi, 1992; Carpene, 1993; Dallinger *et al*., 1997, 2004a, b).

The objective of this study was to determine the distributions of Cd, Cu, Fe, Ni, Pb and Zn in the different parts of the soft tissues and shells of *T. telescopium* which had been collected from Sepang Besar River.

MATERIALS AND METHODS

Snails were collected from Sepang Besar River (N 02º 36' 19.41"; E 101º 42' 11.51") (*see* Fig.1) on 7th January 2006. These samples were brought back to the laboratory for heavy metal analyses. From the visual observation, this sampling site was close to a restaurant, a jetty and a water irrigation facility. The mean height and width of the shells measured in the snails were 8.35 cm and 4.56 cm, respectively. The shells were cleaned by scrubbing in distilled water with a toothbrush to remove biogenic and inorganic particles (Cravo *et al.*, 2004). Meanwhile, total soft tissues of the snail were extracted from the shell and separated into seven different parts (foot, cephalic

TABLE 1
The percentages of weight contributions in the seven soft tissues of *Telescopium telescopium* (N =10).

Soft tissues	%
Digestive caecum	17.33
Foot	15.61
Mantle	8.32
Remaining soft tissues	19.46
Gill	20.07
Cephalic tentacle	5.92
Muscle	13.28

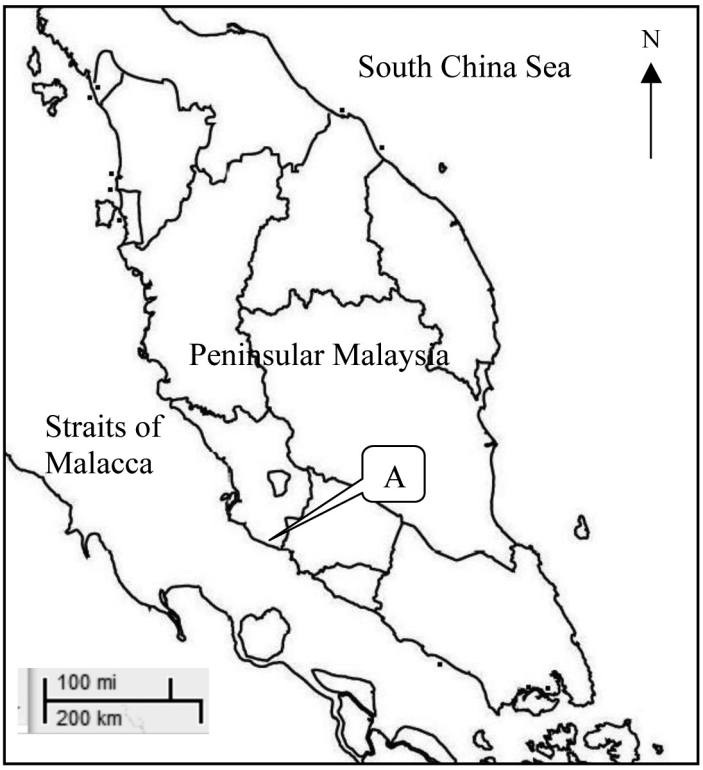

Fig.1: The sampling site of *Telescopium telescopium* at Sepang Besar River (A), Selangor

tentacle, mantle, muscle, gill, digestive caecum and remaining soft tissues) and this were then pooled for each part to form a single batch sample. The percentage of the weight distribution in each separated/ dissected soft tissue is given in Table 1. The shells were separated into three parts (body whorl, middle and apex). All the separated samples were dried at 80°C to constant dry weights. Three replicates of each dissected tissue of the snails were then digested in concentrated nitric acid (BDH: 69%) (Yap *et al.*, 2004). The snail samples were put in a hot-block digester at low temperature (40°C) for 1 h and then fully digested at 140°C for 3hrs (Yap *et al.*, 2002, 2004). The digested samples were added up to 40 ml with double distilled water.

After filtration, the prepared samples were determined for Cd, Cu, Fe, Ni, Pb and Zn by using an air-acetylene flame atomic absorption spectrophotometer (AAS) Perkin Elmer Model AAnalyst 800. The data were presented in µg/g dry weight basis. Multi-level calibration standards were analyzed to generate calibration curves against which the sample concentrations were calculated. Standard solutions were prepared from 1000 mg/L stock solutions of each metal (Merck Titrisol).

All the glassware and plastic materials used were acid-washed in 10%

concentrations of concentrated HCL in order to minimize external contamination. Quality control samples made from standard solutions of Cd, Cu, Fe, Ni, Pb and Zn were analyzed once in every ten samples to check for the metal recoveries. The analytical procedures for the snail samples were also checked with the Certified Reference Material (CRM) for dogfish liver (DOLT-3, National Research Council Canada) and the recoveries of all metal were satisfactory (Table 2).

For the statistical analysis, the distribution of heavy metals in the different parts was determined using the cluster analysis. The relationships between heavy metals in the different parts were analyzed using the Pearson's correlation coefficient. All the data were $\log_{10}$ ($\times$ + 1) transformed prior to the statistical analysis in order to reduce variance (Zar, 1996). SPSS 12.0 was used to conduct the correlation analysis, while STATISTICA 99 edition was used to conduct the cluster analysis.

RESULTS

The concentrations (µg/g dry weight) of six heavy metals in the different soft tissues and three different parts of shells are presented in Table 3. Based on the correlation analysis in Table 4, Cd, Ni and Pb were shown to be positively and significantly ($P< 0.05$) correlated to each other, while Cu, Fe and Zn were found to be positively and significantly ($P< 0.05$) correlated. Most distinctly, Cd, Ni and Pb were negatively (although mostly were not significant) correlated with Cu and Zn. These results indicated that Cu, Fe and Zn are essential metals which are much needed for the basal metabolism of the snails in contrast to Cd and Pb which are non-essential metals and therefore, their binding sites in the cells are different from the essential metals. Although Ni is now recognized as an essential metal in animals, the results obtained in the current work showed that Ni is more correlated to the non-essential Cd and Pb. Nonetheless, further studies are still required to further investigate this particular finding.

Based on the cluster analysis illustrated in Fig.2, the three parts of the shells were separately grouped from the seven soft tissues to indicate that the accumulation, excretion and sequestration of metals are different between the soft and the hard

TABLE 2
A comparison of the metal concentrations (µg/g dry weight) between Certified Reference Materials (DOLT-3 Dogfish-liver) and their measured values.

Metals	Certified values	Measured values	Percentage % of recovery
Cd	19.4 ± 0.600	20.5 ± 0.439	106 ± 2.26
Cu	31.2 ± 1.00	26.5 ± 2.58	85.0 ± 8.28
Fe	1484 ± 57.0	1070	72.1
Ni	2.72 ± 0.350	2.77 ± 0.741	102 ± 27.2
Zn	86.6 ± 2.40	80.9 ± 1.94	93.4 ± 2.24

Note: The certified reference material for Pb is not available.

TABLE 3
The concentrations (µg/g dry weight) of Cd, Cu, Pb, Fe, Ni and Zn in the different soft tissues of *Telescopium telescopium* collected from Sepang Besar River.

Tissues	Pb	Minimum	Maximum	Mean	Std error
Shells	Body whorl	22.6	24.6	23.5	0.58
	Middle shell	23.2	26.4	24.6	0.94
	Apex	20.7	26.7	23.5	1.75
Soft tissues	Foot	0.00	0.56	0.19	0.19
	Cephalic tentacle	0.00	0.28	0.14	0.08
	Gill	14.4	15.9	15.1	0.44
	Muscle	0.00	0.66	0.22	0.22
	Remainder	5.70	8.25	7.06	0.74
	Digestive caecum	9.77	10.9	10.36	0.35
	Mantle	1.21	1.86	1.55	0.19
	Ni	Minimum	Maximum	Mean	Std error
Shells	Body whorl	21.0	22.6	21.7	0.46
	Middle shell	22.2	24.9	23.1	0.89
	Apex	19.7	23.3	21.4	1.06
Soft tissues	Foot	3.74	3.98	3.85	0.06
	Cephalic tentacle	4.80	6.88	5.59	0.64
	Gill	12.3	13.4	12.7	0.36
	Muscle	4.21	6.05	4.95	0.56
	Remainder	8.60	11.2	9.90	0.77
	Digestive caecum	47.9	51.9	50.3	1.18
	Mantle	4.55	5.04	4.84	0.14
	Cu	Minimum	Maximum	Mean	Std error
Shells	Body whorl	6.91	7.36	7.15	0.13
	Middle shell	8.08	9.41	8.95	0.43
	Apex	7.20	8.84	8.21	0.51
Soft tissues	Foot	98.4	111	106	3.98
	Cephalic tentacle	63.6	85.2	76.8	6.66
	Gill	76.1	97.5	86.9	6.18
	Muscle	43.8	58.7	51.1	4.31
	Remainder	66.5	107	88.1	11.9
	Digestive caecum	128	175	147	14.0
	Mantle	81.7	103	90.8	6.36

Table 3 (*continued*)

	Zn	Minimum	Maximum	Mean	Std error
Shells	Body whorl	6.80	7.19	7.02	0.11
	Middle shell	6.98	11.5	8.62	1.46
	Apex	6.22	8.29	7.56	0.66
Soft tissues	Foot	66.3	72.1	69.6	1.72
	Cephalic tentacle	55.3	67.9	60.9	3.70
	Gill	73.7	74.6	74.1	0.27
	Muscle	78.7	83.7	80.5	1.59
	Remainder	47.5	108	84.1	18.6
	Digestive caecum	215	224	220	2.57
	Mantle	65.3	72.0	68.5	1.93
	Cd	Minimum	Maximum	Mean	Std error
Shells	Body whorl	3.18	3.75	3.43	0.16
	Middle shell	3.20	3.47	3.29	0.08
	Apex	2.76	3.22	2.97	0.13
Soft tissues	Foot	0.00	0.02	0.01	0.00
	Cephalic tentacle	0.00	0.06	0.02	0.02
	Gill	0.17	0.65	0.46	0.14
	Muscle	0.04	0.36	0.23	0.09
	Remainder	0.66	0.78	0.72	0.03
	Digestive caecum	2.79	3.11	2.95	0.09
	Mantle	0.40	0.43	0.42	0.01
	Fe	Minimum	Maximum	Mean	Std error
Shells	Body whorl	109	195	153	24.9
	Middle shell	63.2	98.9	76.1	11.4
	Apex	67.0	90.5	77.1	6.97
Soft tissues	Foot	161	224	195	18.3
	Cephalic tentacle	161	217	187	16.1
	Gill	1501	1598	1536	31.2
	Muscle	126	264	201	40.4
	Remainder	826	1316	1147	160
	Digestive caecum	1448	1517	1490	21.2
	Mantle	242	318	279	21.8

Note: Remainder= remaining soft tissues.

TABLE 4
The correlation coefficients of heavy metal concentrations (log_{10} mean +1) based on seven soft tissues and three hard tissues of *Telescopium telescopium* population.

	Pb	Ni	Cu	Zn	Cd	Fe
Pb	1.00	**0.84**	-0.61	-0.56	**0.87**	0.03
Ni		1.00	-0.39	-0.27	**0.91**	0.15
Cu			1.00	**0.97**	**-0.68**	**0.73**
Zn				1.00	-0.58	**0.76**
Cd					1.00	-0.20
Fe						1.00

Note: Values in bold are significant at P> 0.05.

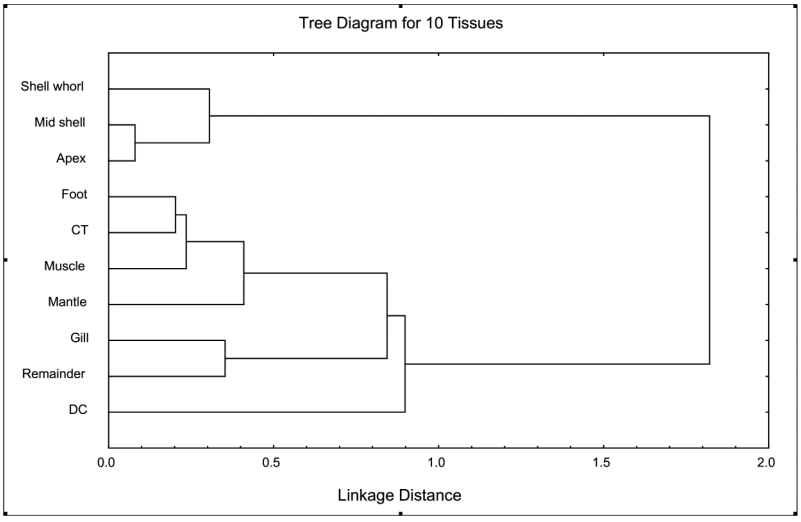

Fig. 2: The clustering pattern of the 10 tissues of *Telescopium telescopium* based on 6 metal concentrations after (log10 [mean +1]) being transformed. Note: CT= Cephalic tentacle; DC = Digestive caecum

tissues. Among the different soft tissues, digestive caecum forms a major cluster differently from the other soft tissues.

DISCUSSION

The discussion of the present data is given based on the following two points.

First, the difference between the clustering pattern in the shells and the soft tissues of T. telescopium.

The metal concentrations found in the shells of the gastropod could be due the different mechanisms of metal accumulations in them. Non-essential metals, like Cd and Pb found in the shell, could be explained by the fact that some trace metals are

incorporated into the shells of the gastropod through substitution of calcium ion in the crystalline phase of the shell or are associated with the organic matrix of the shell (Yap *et al*., 2003a). This is totally different from the binding sites for soft tissues (metallothionein). At first, the metals could be distributed in the different soft tissues before they were biodeposited in the shell of the gastropods (Yap *et al*., 2003a).

This study found that the concentrations of Cd, Ni and Pb in the shells were higher than those in the other soft tissues of the snail. Meanwhile, studies on metal accumulation in shells are useful since they can be used as a record of environmental metal levels (Chow *et al.*, 1976). Shells have important practical advantages over the use of soft tissues for monitoring metal contamination in the aquatic environment since they show less variability (Bourgoin, 1990), integrating elemental concentrations over the life of the molluscs and preserving the metals after the death of the organisms. This could give information about the metal concentrations that they were exposed to in the past (Cravo *et al*., 2004) and offer considerable advantages in preservation and storage. Generally, the metal concentrations in the soft tissues show greater variability than those in the shells (Yap *et al*., 2003a), and this is usually due to seasonal weight changes (associated with physiological conditions, reproductive state) and consequently, shells may provide a more realistic indication of the degree of contamination/pollution (Cravo *et al*., 2002).

Second, the different metal concentrations in the different soft tissues of T. telescopium.

The accumulated metal concentrations were regulated in the different parts of the gastropod soft tissues. In this study, it was found that the different parts (e.g. the digestive caecum and mantle) tended to accumulate high concentrations of heavy metals. Besides, Bebianno and Langston (1995) mentioned that, in general, the tissues where absorption takes place have more metal accumulation than other tissues.

Differential affinities of metals to the binding sites may be associated with different metal accumulations found in the different tissues. In more specific, the high level of certain metal found in a particular tissue might be due to the fact that the metal was tightly bound to the metallothionein, as reported by Roesijadi (1992) in the mussels. The formation of a metal-thiolate complex, with the cysteine residues inside the lysosomes, has caused a slower depuration of the metals found in the different tissues (Yap *et al*., 2003b) which could result in the high level of metals found in the above-mentioned tissues. This mechanism would reduce its toxicity by preventing it from disturbing the cell activities (Webb, 1987).

In addition, the important accumulation of the metals in the different tissues mentioned above could also be related to the functions of these organs. The mantles are in contact with the external medium and are responsible for the metal transfer

to organism. This further indicates that the differences in the surface of contact of the different soft tissues may affect the accumulations of the metals by the mollusc's tissues (Yap *et al*., 2003b). The digestive caecum, which is a part of the digestive gland, plays an important role in heavy metal metabolism, and this thus contributes to their metal detoxification (Viarengo, 1989; Saha *et al*., 2006). This can explain the high metal concentrations in these organs. The different rates of accumulation and the excretion of the metals in the different tissues also result in the different concentrations found in each of the molluscs' tissues (Yap *et al*., 2003b).

The high concentrations of Cu and Zn found in the digestive caecum may be related to the importance of the two metals in the metabolism of foods in the gastropods since Cu and Zn are essential metals. As for the high concentration of Fe found in the operculum, however, it could be due to its essentiality in forming the corneous plate (Ghesquiere, 2005). The different metal accumulations in the different parts of *T. telescopium* were characterized by the accumulativeness of specific metal as revealed in this study, and thus, might allow the accurate estimation of the metal bioavailability in the coastal area. The bioavailability of the contaminants in the environment is a complicated issue which involves many aspects such as chemical, physical and biological (van Straalen *et al*., 2005). Therefore, the use of the different parts of gastropods that are accumulative of specific metal(s) was strongly recommended in the present study.

Generally, the metal concentrations in the digestive caecum are higher compared to other soft tissues (Table 3). This may be due to the crucial role played by the digestive caecum in the animals' nutritional physiology (Menta & Parisi, 2001). The high Cu concentrations found in the remaining soft tissues, mantle and gill might partly be due to hemocyanin (Dallinger *et al.*, 1997). Meanwhile, the metal distribution in the different soft tissues could be due to the environmental metal bioavailability of the habitats and biometric characteristics (Cravo *et al.*, 2004). According to Laskowski and Hopkin (1996), the distribution of metals in soft tissues and shells indicated that contamination in the soft tissues could pose a more important threat to higher trophic levels because the protein in the soft tissues is easily soluble and readily available for higher trophic levels during consumption.

CONCLUSION

The results of the present study indicated the ability of the different soft tissues of *T. telescopium* to accumulate Cd, Cu, Fe, Ni, Pb, and Zn. The cluster analysis showed that metal behaviours for Cd, Ni and Pb were different from Cu, Fe and Zn. Meanwhile, the different clusters between the hard and soft tissues indicated that the binding sites and strategies are different. The present study has also shown that the digestive caecum of *T. telescopium* could be potentially used as a better biomonitoring organ for heavy metal bioavailability and contamination in the intertidal area of Malaysia.

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Potential Co-application of *Burkholderia cepacia*, Calcium and Chitosan on Enhancement of Storage Life and Quality of Papaya Fruits

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ABSTRACT

The fruit of harvested papayas (cv. Sekaki), at colour stage two (mature-green with trace yellow), were treated with fungicide benocide® (0.33 gL^{-1}) or with a combination of *Burkholderia cepacia* B23 (10^9 CFU mL^{-1}) and 0.75% chitosan solution, amended with 3% calcium chloride and stored at 14 ± 0.5ºC and 90-95% RH for 28 d. The effectiveness of the treatments was assessed by evaluating their impacts on storability and changes in the quality attributes of fruits. Results indicated that fruit treated with the combination of *B. cepacia* B23-chitosan-$CaCl_2$ showed delayed climacteric ethylene evolution and reduced respiration rate. The combined treatment reduced weight loss by more than 25% to the control. It also markedly slowed down the ripening of fruits, as shown by their retention of firmness 4.17 N after storage. Moreover, a delayed change in external colour and pH without compromising fruit quality was observed in the fruit receiving the combined treatment. The storage life was thus extended up to 15 d compared to the control. In addition, the incorporation of 3% $CaCl_2$ into the combined treatment significantly increased the calcium content (81%) in the fruit compared to the control, resulting in the improved nutritional value of the papaya. This study provided an alternative method for fungicides treatment of papaya at post-harvest.

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INTRODUCTION

Being a climacteric fruit, papaya (*Carica papaya* L.) is characterized by increased respiration and ethylene evolution during ripening. Generally the fruit ripens in a rather short period between seven to nine days from harvest (Ali *et al.*, 1994). Proper storage practices are required for papaya fruits to avoid quality deterioration which occurs primarily due to post-harvest diseases and accelerated softening. For the fruits to be competitive in the market, it is important to control the disease and to delay the onset of the ripening processes while maintaining the quality.

It is important to note that synthetic fungicides is the primary means used to control post-harvest diseases of fruits; however, environmental and health risks are high (Janisiewicz & Korsten, 2002). Controlled atmosphere (CA) techniques are expensive, while modified atmosphere packaging (MAP) has been shown to ameliorate chilling injury and fungal decay in several crops (Yahia & Paull, 1997). Thus, there is a need to have alternative technique to reduce disease incidence and improve storability of papaya without undesirable physico-chemical changes taking place during the storage. In this sense, post-harvest application of biocontrol agent, in combination with chitosan and calcium chloride, is considered to be an alternative tool.

In our previous study, the antifungal activities of *Burkholderia cepacia* strain B23 were demonstrated in petri plate assays (Kadir *et al.*, 2008; Rahman *et al.*, 2007). The principal mode of disease control of this particular strain is antibiosis. *B. cepacia* has also been shown to protect against or decrease the severity of various post-harvest diseases of fruits, including apples and pears, which are caused by *Penicillium expansum* and *Botrytis cinerea* (Janisiewicz & Roitman, 1988) and banana infection caused by *Colletotrichum musae* (De Costa & Subasinghe, 1999).

Chitosan, which is a high molecular weight cationic polysaccharide, can theoretically be used as a coating material for fruit (Jiang & Li, 2001). Due to its ability to form a semi-permeable film, chitosan coating may be expected to modify the internal atmosphere of fruit and decrease transpiration losses (Zhang & Quantick, 1998). Results of some previous studies have shown that chitosan coating has the potential to prolong storage life and to control decay of many fruit such as strawberries (Hernandez-Munoz *et al.*, 2006), apples (Du *et al.*, 1998) and papaya (Sivakumar *et al.*, 2005).

Calcium has been identified as an important nutraceutical that plays significant roles in the human body to prevent certain diseases (Pszczola, 1998). Many authors have reported that calcium dip increases nutritional value, maintains firmness and extends the storage life of a wide range of fruit, including strawberries and raspberries

(Han *et al.*, 2004), pears (Mahajan & Dhatt, 2004) and peaches (Mahajan & Sharma, 2000).

One of the unique characteristics of chitosan-based coating is that it can be used as a carrier for incorporating functional ingredients, such as antimicrobial agents and nutraceuticals (Park & Zhao, 2004). It can not be denied that works on *B. cepacia*, chitosan and calcium chloride, as post-harvest treatments, are readily available but the literature is still scarce for the local strain of *B. cepacia* and a variety of papayas. Thus, the objective of the study was to determine the potential of postharvest application of *B. cepacia* B23 in combination with chitosan and calcium chloride on the post-harvest storage and quality of papaya fruits under low temperature conditions.

MATERIALS AND METHODS

Preparation of Aqueous Suspension of B. cepacia B23

A local strain of *B. cepacia* B23, isolated from the surface of a papaya fruit, was used as a biocontrol agent in this study. In a previous study, *B. cepacia* B23 was isolated following standard methods and identified using BIOLOG identification system (Rahman *et al*., 2007). To prepare the aqueous antagonist suspension, isolate B23 was grown on nutrient agar (NA) at 28 ± 2ºC for 24 h. A loop of the bacterial culture was then transferred into a 250 mL Erlenmeyer flask containing 50 mL of sterilized nutrient broth (NB) and incubated on a rotary shaker at 150 rpm for 48 h at 28 ± 2ºC. The isolate was re-cultured in fresh NB and incubated for another 72 h before use. At the time of use, the cell concentration of *B. cepacia* B23 in the suspension was adjusted to approximately $1x10^9$ CFU mL^{-1} with sterilized distilled water using spectrophotometer at 600 nm.

Preparation of Chitosan Solutions

To prepare 100 mL of 0.75% chitosan solution, 0.75 g of chitosan (Shrimp shell chitosan, Chito-Chem (M) Sdn. Bhd., Malaysia) was dissolved in 75mL of distilled water added with 2mL of glacial acetic acid. The mixture was heated with continuous stirring for proper dissolution of chitosan. The final pH of the solution was adjusted to 5.6 with 2 N NaOH and volume made up to 100mL with sterilized distilled water. To improve wettability, 0.1mL of Tween 80 was added into the solution (Jiang & Li, 2001).

Fruits and Treatments

Fully matured papayas cv. 'Sekaki' with colour stage two (mature-green with trace yellow) were obtained from an exporter Seng Chew Hup Kee (M) Sdn. Bhd., Kajang, Selongor, Malaysia, on the same day of harvest. Surface sterilization with 75% ethanol was followed by rinsing in sterilized distilled water and air-drying for 10 min for a total of 132 fruit. For one treatment, each of the 44 fruit was dipped for 15 min in (i) sterilized distilled water (control) or (ii) commercial fungicide, benocide® (benomyl 50% WP) of 0.33 g L^{-1}. For the combined treatment, 44 fruit were initially immersed in aqueous suspension of *B. cepacia* B23 (10^9 CFU mL^{-1}) for 15 min and allowed to

air dry for 5 min. Once again, the fruit were immersed in 0.75% chitosan solution which was amended with 3% $CaCl_2$ for 15 min and allowed to surface-dry for 5 min. Each fruit was sleeved with Styrofoam netting, packed in a commercial packaging, and held at 14 ± 0.5 ºC and 90-95% RH for 28 d. Every week, eight fruit (representing four replications for each treatment) were used for the determination of physico-chemical characteristics. A different set of four fruit from each treatment was used to determine the respiration rate and ethylene production, and the same set of fruits was also used throughout the whole storage period. Data were recorded every week, and this was started immediately after the treatment.

Determination of Respiration Rate and Ethylene Production

Respiration rate and ethylene evolution were assayed on a weekly basis. Individual fruit was sealed in a 2.5 L airtight plastic container and incubated for 3 h at 14 ± 0.5ºC. After incubation, one mL of gas sample was withdrawn from headspace by a gas-light hypodermic syringe and analyzed using gas chromatography (Clarus 500, Perkin Elmer, Shelton, USA), equipped with a thermal conductivity detector (TCD), a flame ionization detector (FID) and a Porapack Q, 50/80 stainless steel column. Standard CO_2 and C_2H_4 gasses (Air Products Pte. Ltd., Singapore) were used for calibrating the chromatography. The respiration rate was expressed as mL kg^{-1} h^{-1} of CO_2 evolved, whereas, ethylene production was expressed as µL kg^{-1} h^{-1}.

Measurements of Weight loss, Surface Colour and Flesh Firmness

To determine weight loss, an individual fruit was weighed with a top pan electronic balance (BP2100, Sartorius, Germany) at the beginning of the experiment just after the treatment and then air-dried, and thereafter, this was done each week during the storage period. Eight fruit per treatment (representing four replications) were marked for the measurements of weight loss and surface colour. The same set of fruits was used until the end of the experimental period. Weight loss was expressed as the percentage loss of the initial total weight.

The colour of the surface of papaya was determined using a Chroma Meter (Model CR-300, Minolta Corp., Japan) and expressed in the chromaticity values of lightness (L*), chroma (C*) and hue angle (hº). Before measurement, the equipment was calibrated against a standard white tile, with standard values of L* = 97.30, C* = 1.88, hº = 85.8 using the Illuminate C (CIE 1976). The measurements were taken at stem end, mid region and blossom end on each fruit so as to obtain a mean value.

Meanwhile, pulp firmness of the fruit was measured using an Instron Universal Testing Machine (Model 5543, Instron Corp, USA), which was supported by an Instron Merlin Software Version M12-13664-EN. The instrument was equipped with a 6 mm diameter flat probe that was programmed to penetrate in a normal direction at a cross-head speed of 20 mm min^{-1}. Round slices of 25 mm thick, containing both peel and pulp, were cut horizontally from the stem

end, equatorial and blossom end of each fruit with a razor blade. The measurements were taken at three different places of each slice and the readings were recorded in Newton (N), while the mean was also calculated.

Measurements of Total Soluble Solids, pH, Titratable Acidity and Ascorbic Acid

After the firmness analysis, the pulp tissues of papaya were cut into small pieces. Ten grams of pulp tissues was homogenized in 50 mL of distilled water for 2 min using a kitchen blender and filtered through a Whatman filter paper No. 2. The supernatant was collected in order to measure the total soluble solids using a digital refractometer (Model N-1 α, Atago, Japan), pH using a glass electrode pH meter (GLP 21, Crison, Bercelona, EEC), whereas titratable acidity expressed as citric acid (%) was determined by titration with 0.1 mol L^{-1} NaOH to pH 8.1 according to the method by Ranganna (1977). For ascorbic acid measurement, 10 g pulp tissue was immediately homogenized in 50 mL of 3% cold metaphosphoric acid (HPO_3) using a blender for 2 min, and filtered through Whatman filter paper No. 2. The clear supernatant was collected for assaying ascorbic acid by 2,6-dichlorophenolindophenol titration, following the method of Ranganna (1977). Ten millilitres of aliquot was titrated with 0.1% 2,6-dichlorophenolindophenol solution until the filtrate changed to pink, persisting for at least 15 s and the titration volume of 2,6-dichlorophenolindophenol was recorded. Prior to titration, 2,6-dichlorophenolindophenol solution was calibrated by ascorbic acid standard solution. Ascorbic acid content was calculated according to the titration volume of 2,6-dichlorophenolindophenol and the results were expressed as mg 100 g^{-1} fresh weight.

Calcium Determination

For skin calcium determination, peel with outer flesh of the treated fruits was removed to a depth of 2 mm with a mechanical peeler, and cut into small pieces with a sharp knife. The next 2 mm of the pulp tissue was used for flesh calcium analysis. Each sample was a pooled of peel or flesh from two papayas; four replicates from each treatment were analyzed. The samples were dried in a mechanical convection oven (Memmert, Germany) at 80ºC for two days and ground into powder. Dry ashing procedure was used to digest the powder. The calcium content was analyzed by atomic absorption spectrophotometer (AAnalyst 400, Perkin-Elmer). Calcium measurement was done only at day 0 after the treatment as Ca^{+2} is very stable during storage (Mei *et al.*, 2002); the calcium content is reported in mg kg^{-1}.

Scanning Electron Microscopic Observation of Papaya Fruit Pericarp

Water treated control and *B. cepacia* B23-chitosan-$CaCl_2$ treated papaya fruit were used in this study. The peel samples of ~2 mm^3 were taken from the mid region of the fruit and fixed separately in 2.5% buffered glutaraldehyde for 24 h at 4ºC. The samples were prepared following the standard procedure, as described by Benhamau and

Chet (1996). The samples were dried in a Baltec 030 Critical Point Drying apparatus. The dried samples were stuck on aluminium stubs and coated with gold in a Polaron Sputter Coater and viewed under SEM (JOEL JSM 6400).

Experimental Design and Statistical Analysis

All the experiments were carried out in a completely randomized design (CRD) with three treatments replicated four times. The data were subjected to the analysis of variance (ANOVA) using the SAS statistical software version 8.2. The results showing significant differences were then subjected to mean separation using Tukey's Studentized Range (HSD) Test at $P \leq 0.05$.

RESULTS

Respiration and Ethylene Production

The rate of CO_2 production showed a characteristic of climacteric respiratory pattern occurring during storage at 14 ± 0.5ºC (Fig.1). Immediately after the treatment, the production of CO_2 was found to be higher (7.75 mL kg^{-1} h^{-1}) in the fruit dipped into the combination of *B. cepacia* B23-chitosan-$CaCl_2$, indicating a higher respiration rate than the control (6.69 mL kg^{-1} h^{-1}) or benocide® (6.41 mL kg^{-1} h^{-1}) treated fruits. However, the respiration rate in all the treatments decreased up to 7 d of storage following the initial storage period and then sharply increased in the control and benocide® treated fruit up to 21 d of storage. In the control and benocide® treated fruit, the production of CO_2 reached the maximum levels of 8.23 and 8.42 mL kg^{-1} h^{-1}, respectively on day 21, which were identical to each other. On the other hand, the combination of *B. cepacia* B23-chitosan-$CaCl_2$ suppressed the respiratory production and delayed the onset of the respiratory climacteric. This was markedly lower to the control or benocide® treated fruit. Thus, the

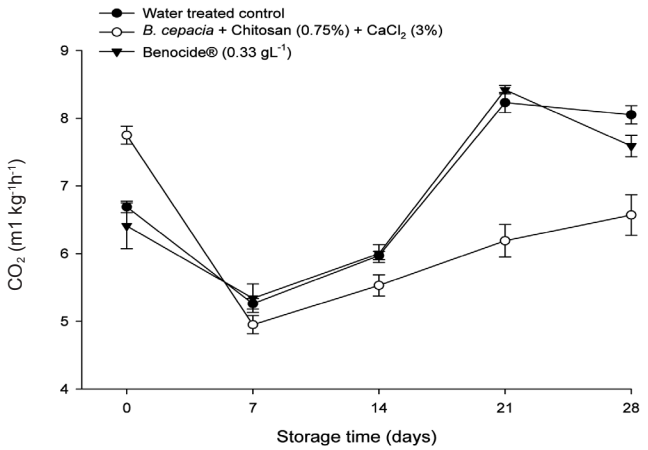

Fig.1: The effect of different treatments on the respiration rate of papayas during storage at 14ºC and 95% RH for 28 d. Each value is the mean of four replications. Means are separated by Tukey's Studentized Range Test at $P \leq 0.05$. Vertical bar represents standard error

combined treatment delayed the respiratory climacteric pattern by almost seven days, as compared to the control or benocide® treated fruit.

As with respiration, ethylene production followed the same climacteric pattern during the storage of fruits for all the treatments (Fig.2). However, the peak was suppressed in the fruit receiving the combination of *B. cepacia*-chitosan-$CaCl_2$. Meanwhile, the fruit under the combined treatment did not produce ethylene up to 14 d of storage. The onset of ethylene production was evidenced after this period, with a substantial increase until the end of storage period with significantly ($P \leq 0.05$) lower rate (0.19 µL kg^{-1} h^{-1}) than the control fruit. Water treated fruit, on the other hand, showed a higher rate of ethylene production after 7 d of storage, and it peaked at 0.59 µL kg^{-1} h^{-1} after 21 d of storage. There were no significant differences in the ethylene production rate throughout the storage period between the control and benocide® treated fruit.

Weight Loss, Surface Colour and Flesh Firmness

Under all the treatments, the papaya fruit showed a progressive loss of weight during four weeks of storage at 14 ± 0.5 ºC and 90-95% RH (Fig.3). However, significantly ($P \leq 0.05$) lower weight loss was consistently recorded with the combination of *B. cepacia* B23-chitosan-$CaCl_2$ dipped fruit as compared to the control or benocide®-dipped fruit. The values ranged between 1.26 to 4.05% for the combined treatment after 7 to 28 d of storage. The control and benocide® treated fruit, on the other hand, exhibited the maximum weight loss at each storage interval with the values 5.46% and 4.81%, respectively after end of storage. No significant differences were observed in the weight loss between the control and benocide®-dipped fruit up to 21 d of storage;

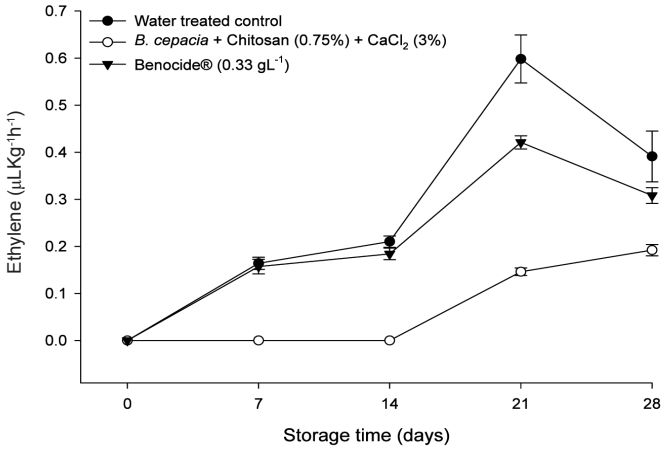

Fig.2: Effects of different treatments on the ethylene production from papayas during storage at 14ºC and 95% RH for 28 d. Each value is the mean of four replications. Means are separated by Tukey's Studentized Range Test at $P \leq 0.05$. Vertical bar represents standard error

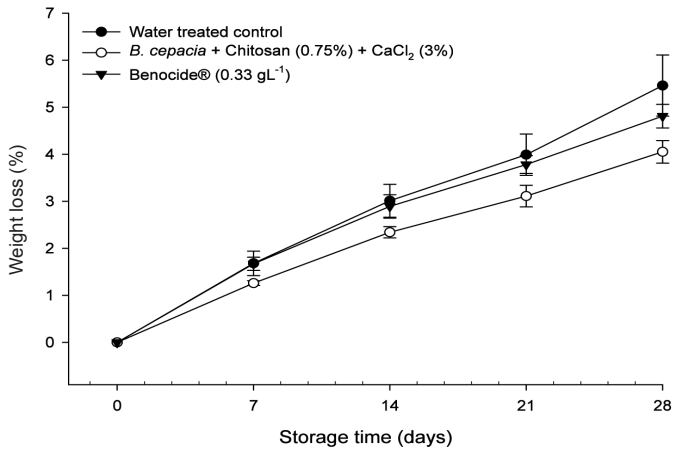

Fig.3: Effect of different treatments on the weight loss of papaya fruits during storage at 14ºC and 95% RH for 28 d. Each value is the mean of four replications. Means are separated by Tukey's Studentized Range Test at $P \leq 0.05$. Vertical bar represents standard error

however, the control fruit showed a higher weight loss than those of other treatments by the end of the storage period.

Scanning Electron Microscopic (SEM) observations showed that *B. cepacia* B23-chitosan-$CaCl_2$ treatment created a film over the fruit surface (Fig.4). The cuticles of the fruit surfaces treated with a combined application were found well arranged with no visible cracks observed (Figure 4A) in relation to the control, whereas, many deep cracks were visible on the epidermal cells of the fruit skin, and cleavages were also apparent (Fig.4B). These cracking on the waxy cuticle and epidermal cells might facilitate water loss from the surface.

The colour changes on the surface of the papaya fruit were monitored by measuring lightness (L*), chroma (C*) and hue angle (h°) during the storage period (Fig.5A-C). The intensity of the green colour of the fruit skin gradually decreased with advancing storage period and this turned to orange-yellow as evidenced by the increasing values of L* and C* of ~48 and ~34, respectively. The fruit under combined treatment consistently exhibited a slower change in the skin colour, as indicated by a more gradual increase in the L* and C* values, ranging from 48.9 to 56.0 and 35.0 to 47.5 respectively after 7 to 28 d of storage. The control fruit, on the other hand, demonstrated the maximum colour changes at each storage interval, as shown by the rapid increases in the L* and C* values, ranging from 54.9 to 63.6 and 46.5 to 59.3, respectively. There were no significant differences between the changes in the L* and C* values in the control and benocide®-treated fruit throughout the evolved storage period.

The initial value of hue angle for all the treated fruit was ~123. Generally, all the fruit showed a significant decrease in their

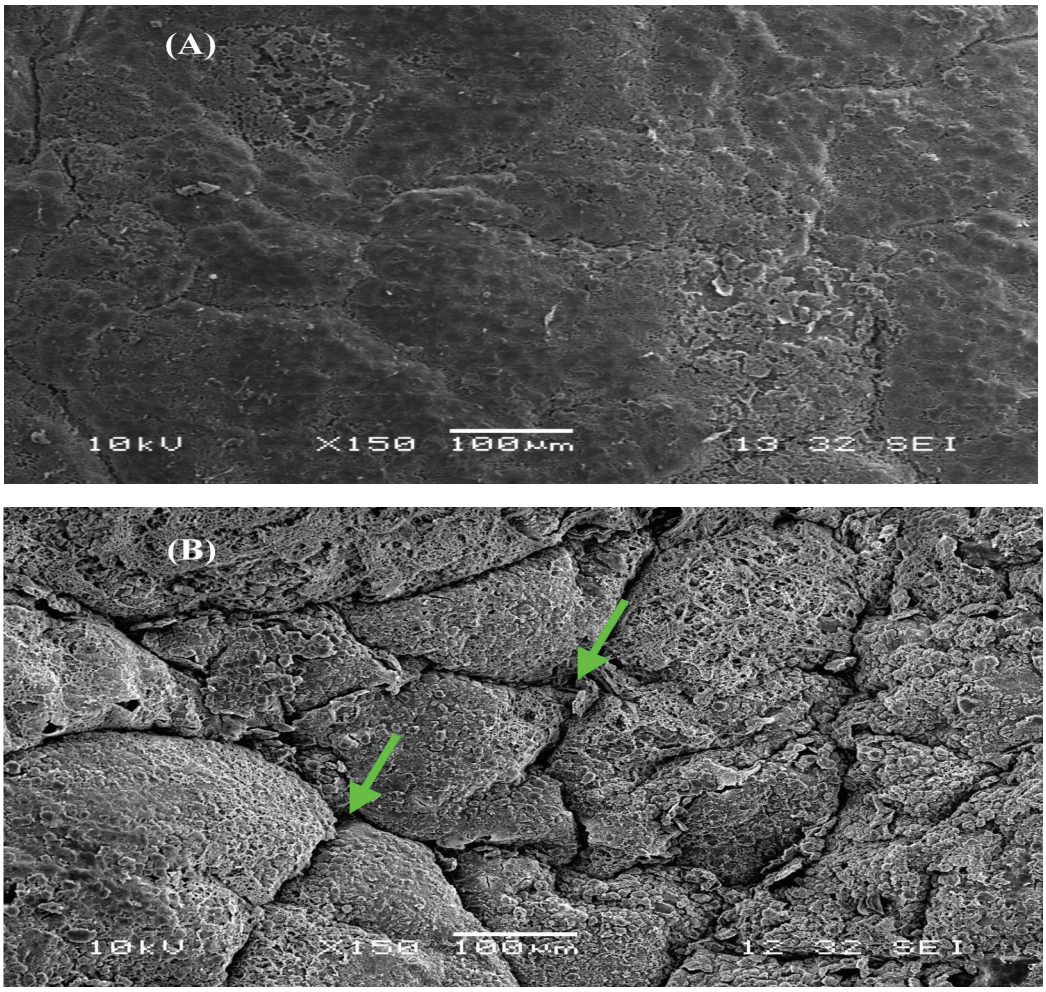

Fig.4: The Scanning Electronic Microscopic (SEM) observations of the fruit pericarp of papaya from *B. cepacia* B23-chitosan-$CaCl_2$ dipped fruit (A) and water dipped fruit (B). Arrow shows deep cracks on the fruit surface

TABLE 1
Calcium content of papaya fruits dipped in benocide® solution or in suspension of *Burkholderia cepacia* B23 incorporated with chitosan and calcium chloride

Treatments	Calcium content (mg kg^{-1})	
	Peel calcium	Flesh calcium
Water treated control	2614.3 ± 31.1 b*	1334 ± 60.35 b*
Benocide® (0.33 g L^{-1})	2575.0 ± 65.5 b	1312 ± 87.5 b
B. cepacia + chitosan (0.75%) + $CaCl_2$ (3%)	6087.5 ± 68 a (132.8%)[1]	2415 ± 76 a (81%)[1]

*Values in each column, followed by the same letter, are not significantly different at $P<0.05$, based on Tukey's Studentized Range Test (HSD).
[1]Values in the parenthesis are the percentage increase in the calcium content over control.

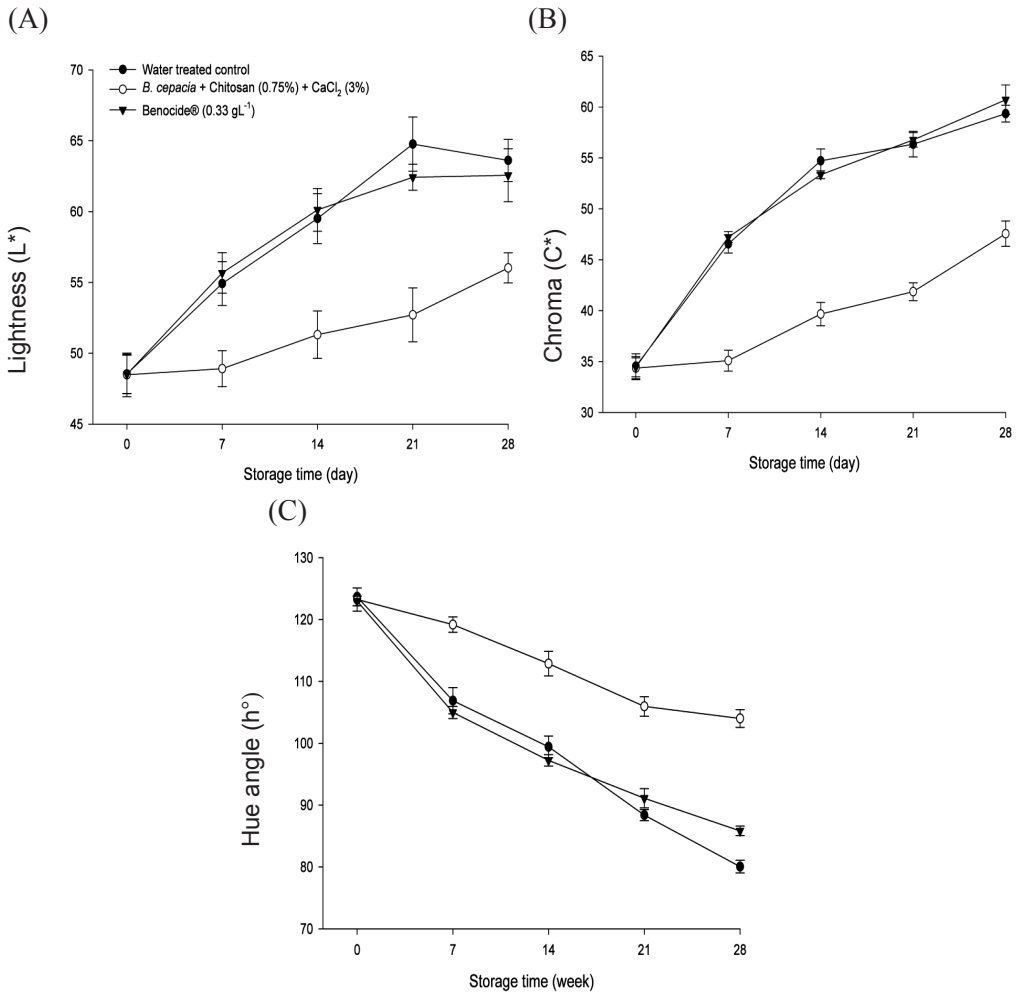

Fig.5: Effects of different treatments on skin colour, lightness (A); chroma (B); hue angle (C) of the papayas during storage at 14ºC and 95% RH for 28 d. Each value is the mean of four replications. Means are separated by Tukey's Studentized Range Test at $P \leq 0.05$. Vertical bar represents standard error

hue angle up to 28 d of storage. At the end of each week of storage, the papaya fruit under the combined treatment exhibited significantly ($P \leq 0.05$) higher h° values, ranging from 119.1 to 104 after 7 and 28 d of storage respectively compared to the control fruit. This indicated a lower rate of colour changes of the skin. In the control fruit, on the contrary, hue angle sharply decreased with storage advanced for which the values were 106.8 to 80 after 7 to 28 d of storage, respectively. A similar trend was also shown by the benocide® treated fruit.

Initially, the firmness of papaya flesh was the maximum (18.7-19.0 N) in all the treatments (fig. 6). The firmness gradually declined for all the fruit, with extended storage period. The rate of the decrease

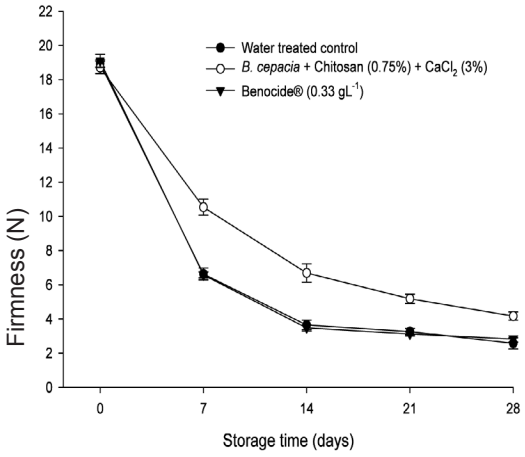

Fig.6: Effects of different treatments on the flesh firmness of papayas during storage at 14ºC and 95% RH for 28 d. Each value is the mean of four replications. Means are separated by Tukey's Studentized Range Test at $P \leq 0.05$. Vertical bar represents standard error

was significantly ($P \leq 0.05$) lower in the fruit subjected to the combined treatment *B. cepacia* B23-chitosan-$CaCl_2$ than those of the control and benocide® treatments. The flesh firmness under the combined treatment was consistently higher than the control or the benocide®-treated fruit during the entire storage period remaining, with the firmness of 4.17 N after 28 d of storage. On the contrary, the control and benocide®-treated fruit manifested sharp decreases in their firmness up to 14 d of storage and thereafter exhibited more or less constant firmness until the end of the storage period. Based on the data on firmness, there was a gain of at least 15 d of extra storage life with the application of the combined treatment. Both the control and benocide®-treated fruit did not show significant differences in term of their firmness throughout the storage period when compared with each other.

Total Soluble Solids, pH, Titratable Acidity and Ascorbic Acid

Changes in the total soluble solids (TSS) content of the papaya fruit during storage are presented in Figure 7. The initial TSS of all the fruit samples was fairly low (~8.2), and this generally increased with ripening. In the control and benocide® treated fruit, the TSS contents reached the maximum level with the values of 12.1 and 11.9, respectively, after 21 d of storage, and these were significantly ($P \leq 0.05$) higher than that of the fruits treated with the combination of *B. cepacia* B23-chitosan-$CaCl_2$. After this period, noticeable decrease in the TSS was recorded in the control and benocide® treated fruit. In contrast, the fruit under the combined treatment showed a gradual improvement in TSS content registering the maximum value of 10.88 at the end of storage period. This showed that the fruit

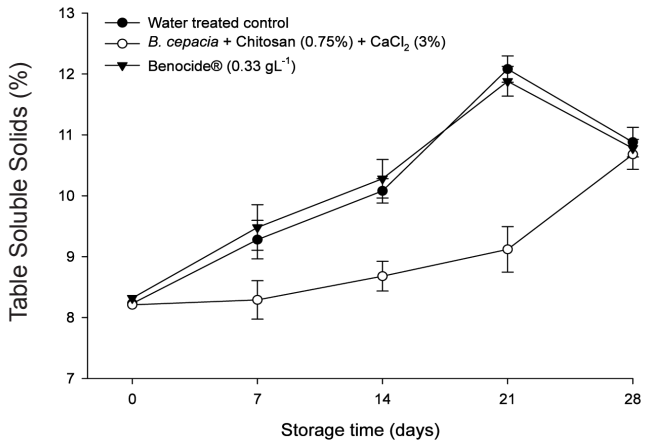

Fig.7: Effects of different treatments on total soluble solids of papayas during storage at 14ºC and 95% RH for 28 d. Each value is the mean of four replications. Means are separated by Tukey's Studentized Range Test at $P \leq 0.05$. Vertical bar represents standard error

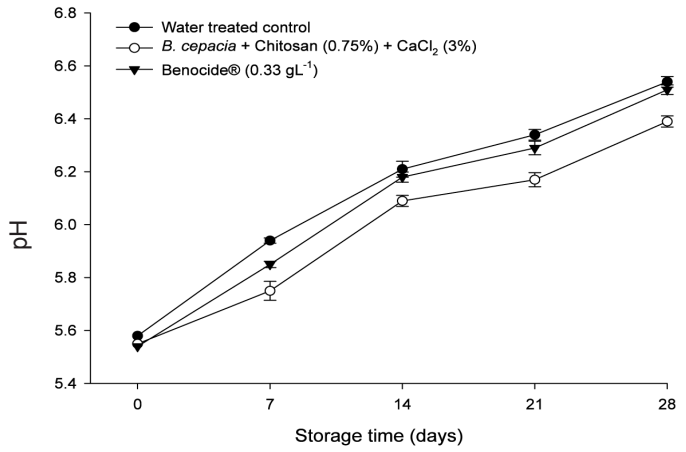

Fig.8: Effects of different treatments on the pH of papayas during storage at 14ºC and 95% RH for 28 d. Each value is the mean of four replications. Means are separated by Tukey's Studentized Range Test at $P \leq 0.05$. Vertical bar represents standard error

had not reached the full ripening stage for them to be immediately marketable.

The changes in the pH value of papaya, as a function of different treatments and storage time, are shown in Fig.8. The pH value of fruit gradually increased as storage progressed with significant differences ($P \leq 0.05$) between the treatments. At the end of a storage period of 28 d, the pH value was significantly lower (6.3) in the fruit that were subjected to the combination of *B. cepacia* B23-chitosan-$CaCl_2$ to the control fruit (6.5). Nonetheless, no significant variation was observed in the pH values of the control

and the benocide®-treated fruit throughout the storage period.

It is evident that the combination of *B. cepacia* B23-chitosan-$CaCl_2$ induced a significant variation in the ascorbic acid content of the fruits during storage (Fig.9). Initially, the ascorbic acid content was ~52 mg 100 g^{-1} for all the treatments. With the control fruit, however, the content sharply increased over time and reached the maximum value of 72.5 mg 100 g^{-1} on day 14 but it declined until the end of storage thereafter. A similar trend was observed for the benocide®-treated fruit. On the contrarily, the fruit subjected to the combined treatment showed a more gradual decline with time and exhibited the maximum value of 64.7 mg 100 g^{-1} after 21 d of storage, but slightly declined thereafter.

Fruit Calcium Content

As expected, fruit treated with the combination of *B. cepacia* B23-chitosan-$CaCl_2$ resulted in significantly ($P \leq 0.05$) higher calcium contents of 6087.5 and 2415 mg kg^{-1} in the peel and flesh tissues, respectively, as compared to that those found in the control or Benocide®-treated fruit (Table 1). The addition of 3% $CaCl_2$ into the chitosan solution increased the content of Ca^{+2} by 132.8 and 81% in the peel and flesh tissues, respectively as compared to the control.

DISCUSSION

Generally, climacteric fruit exhibits a rapid rise in respiration rate at the onset of ripening, which subsequently slows down as the fruit ripens (Sirivatanapa, 2006). Thus, the storage life of climacteric fruit is usually shorter than that of non-climacteric fruit. Likewise, in this study, the papaya fruit exhibited a respiratory climacteric, which appeared simultaneously with an increase in the ethylene synthesis. The application of *B. cepacia* B23, in combination with

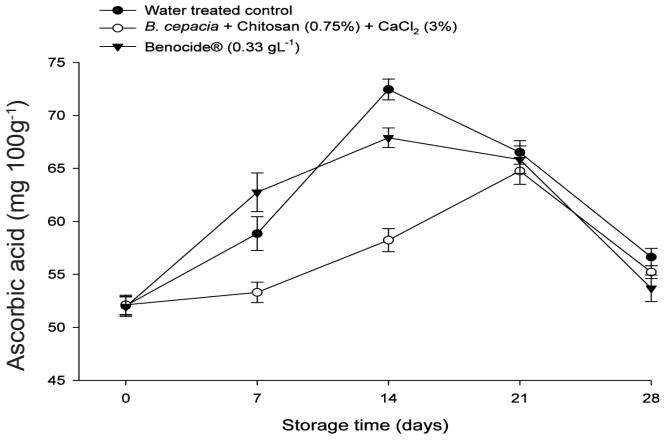

Fig.9: Effects of different treatments on the ascorbic acid content of papayas during storage at 14ºC and 95% RH for 28 d. Each value is the mean of four replications. Means are separated by Tukey's Studentized Range Test at P≤0.05. Vertical bar represents standard error

calcium incorporated-chitosan coating on the papaya fruit as a post-harvest treatment, showed beneficial effects on the respiration rate, ethylene production, weight loss and loss of firmness. Moreover, in the previous study, this combined treatment exhibited a good control of anthracnose on artificially inoculated and naturally infected papaya fruit (Rahman *et al.*, 2009).

The chitosan-based coating can form a protective barrier on the surface of fresh fruit, reduce water loss, inhibit gas exchange, decrease nutrient loss, and prevent micro-organism growth that causes fruit rotting (Qiuping & Wenshui, 2007). In this study, the combined treatment was found to significantly reduce the respiration rate, ethylene production and weight loss. The effectiveness of this particular treatment might be due to the biological activity of *B. cepacia* B23 and the filmogenic properties of chitosan-$CaCl_2$. In this case, chitosan acted as a carrier of *B. cepacia* B23 and $CaCl_2$, together with its coating capability, which modified the atmospheric compositions inside the fruits. Since an inhibition of CO_2 evolution was the consequence of bioactive coating, ethylene production of the fruits would also be reduced (Bautista-Banos *et al.*, 2006). Such inhibitory effects on both the respiration and ethylene productions were reported in tomatoes and peaches coated with chitosan (El Ghaouth *et al.*, 1992; Li & Yu, 2000). Immediately after the treatment, the papayas that were subjected to the combination of *B. cepacia* B23-chitosan-$CaCl_2$ exhibited an increased respiration rate, probably because of an induced stress of the acetic acid solution (Devlieghere *et al.*, 2004). In an earlier study, El Ghaouth *et al.* (1991) observed an immediate stimulation of the respiration in the chitosan coated strawberry, and it disappeared gradually.

The role of *B. cepacia* B23 in reducing respiration rate and ethylene production was not very clear; however, this bacterium might directly assist in the removal of ethylene from the fruit surroundings by using it as a biochemical substrate (Reid, 1992). Moreover, in our previous study, it was found that *B. cepacia* significantly reduced the anthracnose disease in papaya fruit (Rahman *et al.*, 2009), and this might be associated with reduced respiration and ethylene production rate through by controlling the infection. This contention is in agreement with some previous researchers, who have reported that the degree of microbial spoilage of fresh-cut honeydew and cantaloupes is correlated to the increase in the respiration rate (Luna-Guzman & Barrett, 2000; Saftner *et al.*, 2003). Thus, any reduction of disease infection will eventually lead to lower rates of respiration and ethylene synthesis.

Surface coating with chitosan-based matrix was reported to reduce weight loss of various fruit types, such as strawberries and raspberries (Hernandez-Munoz *et al.*, 2006), longan (Jiang & Li, 2001) and papayas (Sivakumar *et al.*, 2005). In this study, the combined treatment of *B. cepacia* B23-chitosan-$CaCl_2$ significantly reduced the weight loss of fresh papaya fruit during storage at 14ºC for 28 d. Due to its ability

to form a semi-permeable coating around the fruit, chitosan reduces the weight loss by controlling the migration of water vapour through the surface of fruit. The anti-fungal and moisture barrier functions of chitosan-based coating were not altered by the incorporation of 3% $CaCl_2$ into the treatment (Han *et al.*, 2004). Meanwhile, the beneficial effect of $CaCl_2$ in reducing post-harvest weight loss has been reported in Asian pear (Mahajan & Dhatt, 2004). The authors explained that the reduction in weight loss was attributed to the influence of calcium in maintaining the firmness of the fruit and tissue rigidity, thereby checking moisture loss from fruit.

Loss of firmness is one of the major factors limiting the post-harvest quality and storage life of fruit and vegetables. In the present study, better firmness was attributed to the papaya fruit subjected to the combination of *B. cepacia* B23-chitosan-$CaCl_2$. In this combined treatment, chitosan coating reduced weight loss, while slowing down the migration of water vapour from the fruit surface and thus, controlling the integrity and texture of cells, resulting in the maintenance of firmness (Hernandez-Munoz *et al.*, 2006), which was further enhanced by the incorporation of calcium. The firming effect with the incorporation of 3% $CaCl_2$ was expected as calcium plays an important role in stabilizing cell membrane through the formation of calcium pectates, which might increase the rigidity of cell wall and the middle lamella of the fruit (Picchioni *et al.*, 1996) and therefore, maintaining cell turgor potentials (Mignani *et al.*, 1995). Hence, the application of *B. cepacia* B23 with chitosan-$CaCl_2$ probably has a synergistic or additive effect in maintaining the firmness of papaya. This result is in agreement with that of Han *et al.* (2004) who found the highest firmness in strawberries and raspberries treated with chitosan containing higher concentration of calcium.

Generally, the external colour of fruit is retained when coated with chitosan solution (Bautista-Banos *et al.*, 2006). In this study, the extent of skin colour development of the papaya fruit was significantly slowed down when treated with the combination of *B. cepacia* B23-chitosan-$CaCl_2$ compared to the control. Meanwhile, the application of the combined treatment formed a semi-permeable film, which caused modification of gaseous compositions around the interior of fruit surface and consequently reduced respiration rate and ethylene production and action (Kader *et al.*, 1989). These conditions delayed ripening and senescence process, resulting in retention of green colour and firmness of fruit. The results of this study support the findings by Sivakumar *et al.* (2005) who found that chitosan coating amended with ammonium chloride retarded colour development of skin and the flesh of papaya during storage. Since attack by pathogens is a major factor causing discoloration of harvested fruit (Jiang *et al.*, 2005), the delay in the skin colour development by the combination of *B. cepacia* B23-chitosan-$CaCl_2$ could be partially beneficial due to the control of decay in this study. This result is in concordant with the work of Jiang and Li

(2001) who noted that inhibiting decay by chitosan coating resulted in the delay in skin colour changes of longan fruit.

TSS, ascorbic acid, titratable acidity and pH are important quality parameters of papaya. The results of the current study showed that the treatment with the combination of *B. cepacia* B23-chitosan-$CaCl_2$ exhibited a beneficial effect on the changes in the quality of papayas during storage. This combined treatment slowed down the accumulation of TSS and ascorbic acid, and reduced the change in pH of fruit during storage. This could be due to the reduction of oxygen supply on the fruit surface which resulted in a lower respiration rate and the growth inhibition of spoilage organisms (Yonemoto *et al.*, 2002). The results of this study are in agreement with the findings of the previous works on various fruit coated with chitosan-based coatings, such as Indian jujube (Qiuping & Wenshui, 2007) and mangoes (Srinivasa *et al.*, 2002).

The addition of 3% $CaCl_2$ into the combined treatment enriched Ca^{+2} content in the papayas, where flesh Ca^{+2} content was increased by 81% as compared to the control fruits, and thus, resulting in an increased nutritional value of the fruit. The results of the current work further strengthened the findings by Han *et al.* (2004) who reported that chitosan-based coatings containing calcium or vitamin E significantly increased the content of these nutrients in both fresh and frozen strawberries and raspberries during storage.

CONCLUSION

The combination of *B. cepacia* B23-chitosan-$CaCl_2$ extended the storage life of papaya by inhibiting its respiration rate and ethylene production. It reduced weight loss and delayed changes in colour and pH during storage without impairing the quality of the fruit. In addition, chitosan-based coating demonstrated its potentiality to carry microbial antagonist and high concentration of $CaCl_2$, which thus significantly increased the content of calcium in papayas. It is therefore obvious that the combination of *B. cepacia* B23-chitosan-$CaCl_2$ has the potential to improve storability and enhance the nutritional value of fresh papayas and can be commercially used as a post-harvest treatment.

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Population Genetics of the Cave-dwelling Dusky Fruit Bat, *Penthetor lucasi,* Based on Four Populations in Malaysia

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ABSTRACT

The population genetics of *P. lucasi* was inferred using 1,061 base pairs (bp) of the Cytochrome *b* mitochondrial gene. A total of 77 individuals were classified *a priori* according to their localities, namely, Miri, Kuching, Sri Aman and Kelantan. Results showed that the populations of *P. lucasi* were separated into two haplogroups, namely, Haplogroup 1 (found in Miri and Kuching populations) and Haplogroup 2 (Miri, Kuching, Sri Aman and Kelantan populations). This separation was supported by bootstrap values in the phylogenetics analyses (94.9% in the maximum likelihood and 100% in Bayesian). A high level of genetic divergence was detected between two haplogroups (3.88%) and this separation could be related to historical events which include multiple colonisation and Pleistocene refugia during the Last Glacial Maximum ice age period. High genetic divergence within Miri (4.93%) and Kuching (4.72%) populations could be due to the presence of a species complex within the *P. lucasi* populations. The presence of haplotypes from both the populations in Haplogroup 1 and Haplogroup 2 might be due to the ability of this particular species of bats to perform long-distance flight for foraging. A high gene flow between these populations suggests a widespread female gene flow of *P. lucasi,* judging from the distance of both localities. Meanwhile, the absence of a deep structure from the haplotype trees further proves that *P. lucasi* may have had a wide dispersal ability since the Pleistocene has allowed for genetic exchange to occur between the regions in Malaysia.

Keywords: Penthetor lucasi, population study, genetic diversity, mitochondrial DNA

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INTRODUCTION

An understanding of a species population structure typically provides significant

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information to address questions relating to both past and present evolutionary and behavioural processes of organism. Thus, the introduction of molecular techniques is a great breakthrough in the pursuit of such understandings. This is especially true for studies in which traditional methods, such as the direct observation of individuals or populations, are greatly restricted (Burland & Worthington-Wilmer, 2001). Numerous studies on intraspecific phylogenetics and phylogeography of organisms have also positively impacted the current level of knowledge of species evolution and speciation.

The use of genetic markers has led to the description and a better understanding on social life (Bryja *et al.,* 2009). Today, studies on population genetics in bats have further revealed that phylogeographic variations are affected by various factors, such as seasonal migrations, geographical barriers, and past processes (Burland & Worthington-Wilmer, 2001; Bryja *et al.,* 2009). In the Indo-Malayan region, such studies have been conducted by various authors (e.g. Kitchener *et al.*, 1993a, 1993b; Schmitt *et al.*, 1995; Hisheh *et al*., 1998; Abdullah, 2003; Mahadatunkamsi *et al*., 2003; Imelda, 2007; Tingga, 2010). Other than bats, population genetics studies on other taxa in this region have also been documented, including on birds (Rahman, 2000), fish (Esa *et al.,* 2008) and frogs (Ramlah, 2009). These studies have utilised various genetic markers, such as allozymes, RNA, mtDNA and nuclear DNA.

Isolation is one of the major factors facilitating evolutionary changes. A cave is a good example of habitat isolation, which is surrounded by mosaic habitat types. However, the presence of gene flow between populations over long distances will decrease differentiation, and it is assumed that genetic structuring is weak across the macrogeographical range in migratory bats (McCracken *et al.,* 1994; Webb & Tidemann, 1996; Hisheh *et al.,* 1998; Russell *et al.,* 2005). In contrast, the non-migratory ghost bat (*Macroderma gigas*) shows a clear genetic structuring among the populations in Australia (Worthington-Wilmer *et al.,* 1994).

The dusky fruit bat or *Penthetor lucasi* was selected for this study as it is known to live specifically near total darkness in isolated caves. This particular species has gone through several taxonomic reviews from *Cynopterus* (*Ptenochirus*) *lucasi* Trouessart (1897) to *Ptenochirus lucasi* Trouessart (1904), and is presently placed in the genus *Penthetor* (Andersen, 1912; Maryanto, 2004). This bat is medium in size, with dark grey brown upperpart and pale buffy underpart. Sometimes, the specimens are observed to have a distinct dark shade at the centre of the head and paler near the eyes. It is widely distributed throughout the southern part of Thailand, Peninsular Malaysia, the Riau Archipelago, Borneo (Payne *et al.,* 1985; Corbet & Hill, 1992; Abdullah *et al.,* 2007; Francis, 2008; Abdullah *et al*., 2010) and Sumatra (Maryanto, 2004). A morphological

study on the species in Sarawak showed differences in the body and skull sizes (Sri Aman, Kuching and Miri populations). It was suggested that different ecological factors, such as breeding, crowding effect, foraging behaviour, resource availability and selective pressure, are the possible causes of the morphological variation among *P. lucasi* populations (Abd Rahman & Abdullah, 2010).

This study aimed to examine the phylogenetic relationships, diversification and genetic variation within the *P. lucasi* populations in Malaysia, inferring from the mtDNA Cytochrome *b* (Cyt *b*) gene. It was hypothesised that *P. lucasi* had high site fidelity for roosting. Thus, there would be low gene flow and high genetic divergence among the isolated roosts in Malaysia.

MATERIALS AND METHODS

Samples Collection and DNA Extraction

A total of 77 individuals of *P. lucasi* from four populations, namely Miri (33 individuals), Kuching (33 individuals), Sri Aman (six individuals) and Kelantan (five individuals), were used in this study (see Figure 1). The specimens were collected using mist nets and then euthanized using chloroform, and preserved in 95% ethanol prior to genetic analysis. Museum samples from the zoological collections at Universiti Malaysia Sarawak (Abdullah *et al.,* 2010) and the Department of Wildlife and National Park or DWNP (Pahang) were also included in this study. All the specimens used are listed in **Appendix 1.** DNA extraction was done using the cetyltrimethylammonium bromide (CTAB) method (Grewe *et al.,* 1993), with the presence of proteinase K. Extracted DNA was visualized on 1% agarose gels containing ethidium bromide, run for approximately 30 minutes at 90 V, and then photographed under ultraviolet (UV) illumination. The isolated DNA was used for further mtDNA analyses.

Polymerase Chain Reaction (PCR) and DNA Sequencing

Approximately 1061 base pairs (bp) of Cyt *b* were amplified following the standard protocol as described by Sambrook *et al*. (1989). A pair of Cyt *b* primers were used, 5'-CGAAGTTGATATGAA AAACCATCGTTG-3', and known as L14724 (forward) (Irwin *et al.,* 1991) and 5'-AACTGCAGTCATCTCCGGT TTACAAGAC-3' known as H15915 (reverse) (Irwin *et al.,* 1991). A total volume of 25 µl master mix was made comprising of 5.0 µl 5X colourless GoTaq® Flexi buffer, 1.5 µl of $MgCl_2$ solution (25 mM), 0.5 µl of dNTP mix (10 mM), 1.0 µl of each forward and reverse primers (10 mM) 15.5 µl of deionised distilled water, 1.0 µl of DNA template and 0.5 µl GoTaq® DNA polymerase (5u/µl). PCR was carried out using a thermocycler with 30 cycles inclusive of one initial denaturation at 94ºC and final extension at 72ºC for three and five minutes, respectively. The other 29 cycles consisted of denaturation at 94ºC for one minute, annealing at 40ºC for one minute and an extension at 72ºC for two minutes. Amplification products were then visualised using the agarose gel electrophoresis

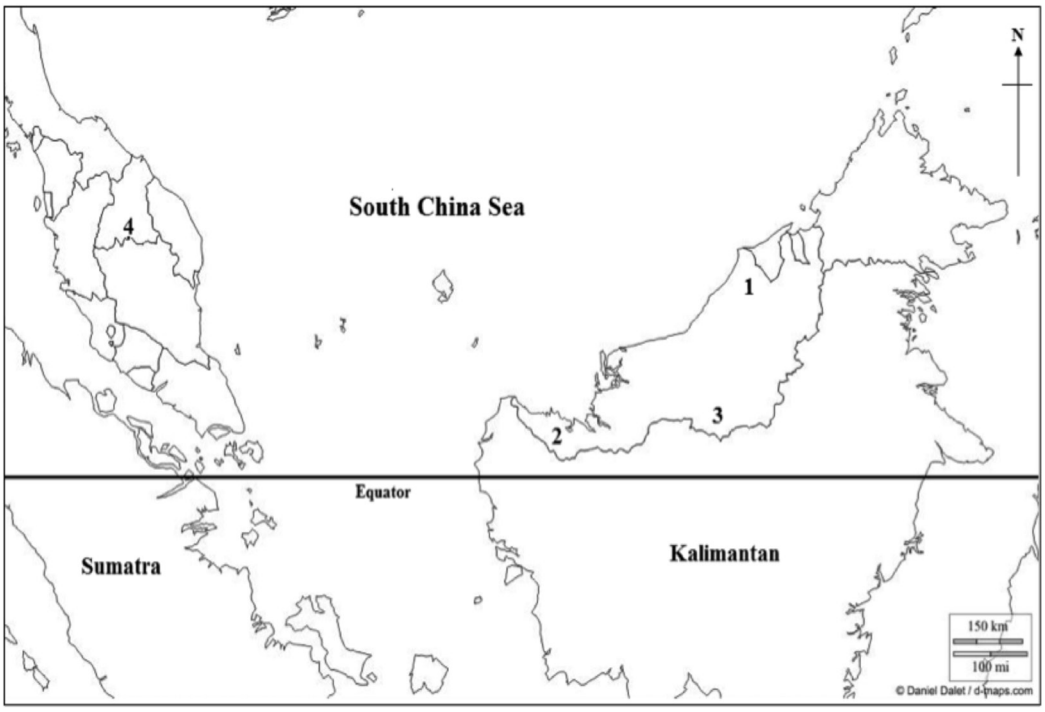

Fig. 1: Maps showing the type locality of *P. lucasi* specimens used in the molecular analyses; 1- Miri; 2 - Kuching; 3 - Sri Aman; 4 - Kelantan. Map was modified from Dalet (2010).

method. DNA Purification was done using the Promega Wizard SV Gel and PCR Clean Up System (Promega Co.). The purified samples were then sent for sequencing at a private laboratory using ABI prism ™ Big dye ™ terminator cycle sequencing Ready Reaction Kit version 3.1 or using the ABI PRISM ® 377 DNA Sequencer with the BigDye®Terminator v3.0 Cycle Sequencing Kit. The sequencing product was run using ABI 3730 XL capillary DNA sequencer (50 cm capillary).

Sequence Alignment and Phylogenetic Analyses

The DNA sequence results were displayed using the CHROMAS version 1.45 software (McCarthy, 1996). The multiple alignments of DNA sequences were done using CLUSTAL X (Thompson *et al.*, 1997) software. The pair-wise distance between the populations were computed using the Molecular Evolutionary Genetic Analysis (MEGA) software version 3.0 (Kumar *et al.*, 2004), with correction using a Kimura 2-parameter (K2P) model (Kimura, 1980). The time of divergence of bats was estimated following Brown *et al.* (1982), which was based on an evolutionary rate of Cyt *b* gene at 2% substitution rate per million years and calculated using Kimura-2 parameter distance matrix implemented in MEGA version 3.0 (Kumar *et al*., 2004).

A maximum likelihood (ML) tree was constructed by using phylogenetics analysis using Parsimony (PAUP) version 4.0beta

(Swofford, 1998), whereas a Bayesian tree was constructed using MrBayes version 3.0 (Huelsenbeck & Ronquist, 2001). The Akaike Information Criterion (AIC) was used to determine the best-fit-model of sequence evolution in the species by using Modeltest 3.7 (Pasoda & Crandall, 1998). The Maximum Likelihood (ML) and Bayesian trees were constructed based on the General Time reversible (GTR) model (Tavare, 1986), as determined by AIC. For ML, the heuristic search option was used in PAUP* with Tree-bisection-reconnection (TBR) branch swapping and 10 random additional sequence replicates. The Bayesian analysis was performed with 2 745 000 generations implementing Metropolis-coupled Markov chain Monte Carlo (MCMC) with 100 generation and burn in=1000 for summary parameter values and trees. The trees were rooted with two outgroups, namely, *Cynopterus brachyotis* (TK152458; Abd Rahman, 2010) and *Rhinolophus philippinensis* (TK152938; Abd Rahman, 2010). To obtain a graphical representation of the Cyt *b* gene variation, minimum spanning networks (MSN) of haplotypes were constructed by allowing all the required mutational steps that would eventually link the different sub-networks. These haplotype networks were generated using the programme, Network 4.5.0.2 (Fluxus Technology 2004-2008).

Population Genetic Analyses

Haplotype (*h*) and nucleotide (π) diversities (Nei & Tajima, 1981; Nei, 1987), nucleotide divergence (Da), the number of polymorphic sites (S) and the mean number of nucleotide differences (K) were calculated using the DnaSP version 4.5 (Rozas *et al.*, 2003). The Mantel test was conducted in Arlequin Version 3.0 (Exoffier *et al.,* 2005). Permutations of size 1000 were used to examine the effect of isolation-by-distance (IBD) by testing the correlation between geographical distance and genetic differentiation among the populations. The neutrality tests of Tajima's, *D* (Tajima, 1989), Fu and Li's *D** and *F** (Fu & Li, 1993) and Fu's F_s (Fu, 1997) were used to test the hypothesis that all mutations are selectively neutral (Kimura, 1983). Tajima *D* is based on the differences between the number of segregating sites and the average number of nucleotide differences (Tajima, 1989). Fu and Li's *D** and *F** tests are based on molecular polymorphism data (Fu & Li, 1993). Fu's F_s (Fu, 1997) assessment of the haplotype structure on the haplotype frequency distribution was used as an additional neutrality test. The level of population subdivision (F_{st}) (Hudson *et al.,* 1992), nucleotide subdivision (N_{st}) (Lynch & Crease, 1990), and the number of female migrant (N*m*) (Hudson *et al.,* 1992) for determining the gene flow were calculated using DnaSP version 4.5 (Rozas *et al.*, 2003). The analysis of Molecular Variance (AMOVA) was used to estimate F-statistic (Φ_{st}) (Weir & Cockerham, 1984) values in order to assess further differentiation among the populations. The significance was tested using 10 000 permutations, as performed using the Arlequin Version 3.0 software (Excoffier *et al.,* 2005).

RESULTS

Analysis of Sequence

A total of 1,061 bp of cyt *b* of 77 *P. lucasi* individuals were successfully sequenced. Out of the total, 95 were variable sites (8.95%) comprising 28 singleton sites (29.47%) and 67 parsimony informative sites (70.53%). On the average, the nucleotide composition consisted of adenosine (A) = 29.6%, thymine (T) = 24.3%, cytosine (C) = 32% and guanine (G) = 14.1%. The overall frequency distributions of nucleotides at the first, second and third codon positions [values in percentages (%); A = 26.1, 20.1, 42.6, T = 23.0, 41.2, 8.7, C = 27.0, 24.6, 44.3 and G = 23.9, 14.1, 4.3]. All the sequences were submitted to the GenBank with the accession numbers GU724879-GU724957.

Haplotypes Distribution of P. lucasi

Haplotype trees of *P. lucasi* were constructed using the maximum likelihood (ML) and the Bayesian methods (see Fig.2 and Fig.3). Generally, both trees showed the same grouping of *P. lucasi*, with only slight differences in their topology. These trees revealed the monophyly of *P. lucasi* (94.9% ML of bootstraps support; and 100% in BPP) with respect to the out-groups, *C. brachyotis* and *R. philippinensis*. Two clades were constructed from the phylogenetics trees, namely, Haplogroup 1 and Haplogroup 2. Haplogroup 1 comprised 31 haplotypes of *P. lucasi* from Miri and Kuching, while Haplogroup 2 consisted of 14 haplotypes of *P. lucasi* from Miri, Kuching, Sri Aman and Kelantan.

Haplotype Network

The phylogenetic structure among the samples from the four populations of *P. lucasi* was revealed by haplotype clustering on a minimum-spanning network (MSN) (Fig.4). Based on the unrooted network of mtDNA cyt *b*, the MSN showed a 'star-like' phylogeny in the *P. lucasi* populations in Malaysia. Furthermore, the MSN topology pattern is similar to other haplotype trees (ML and Bayesian), which include two groups of sequences from the populations of Miri-Kuching (Haplogroup 1) and Kuching-Miri-Sri-Aman-Kelantan (Haplogroup 2), respectively. Within both sub-networks, most of the haplotypes were

TABLE 1
Number of haplotypes and nucleotide diversity within each population of *P. lucasi*.

Localities	N	No. of haplotypes	Haplotype diversity (*h*)†	Nucleotide diversity (π)*†	% Pairwise divergence*†
Miri	33	26	0.985 ± 0.011	0.01584 ± 0.00321	0.00 - 4.72
Kuching	33	17	0.938 ± 0.023	0.01316 ± 0.00343	0.00 - 4.93
Sri Aman	6	3	0.733 ± 0.155	0.00082 ± 0.00023	0.00 - 0.19
Kelantan	5	4	0.900 ± 0.161	0.00528 ± 0.00105	0.00 - 0.76

N=Number of individuals
*Estimated using Kimura two-parameter distance (Kimura, 1980)
†Sites with gaps were completely excluded.

unique to individuals (30/45), while 15 haplotypes were associated with more than one individual. Haplotype frequencies were denoted by the proportional size of haplonodes. Thirty-seven mutational steps link the two haplogroups.

Both the haplogroup sub-networks were rather complex with divergent branches marked with grey nodes, indicating hypothetical haplotypes (missing haplotypes). Within haplogroup 1, five haplotypes (namely, haplotypes 1, 10, 12, 13 and 25) were shared between Miri and Kuching populations, with a high frequency suggesting the female gene flow. All the haplotypes from Miri and Kuching populations were divergent with the mutational step ranging from one to four. Within haplogroup 2, the Miri population diverged by one to five mutational steps. The Kuching population was divergent with mutational steps ranging from one to three, while the Kelantan population diverged by one to four mutational steps. All Sri Aman haplotypes were divergent with a single mutational step.

Nucleotide Divergence within and among the Populations

A total of 95 segregating sites were detected from 45 haplotypes that were distributed within and among the four populations of *P. lucasi*. From the total of 77 individuals, six haplotypes were shared between the populations, namely; H1, H10, H12, H13 and H25 and all were shared between Miri and Kuching. The population from Miri showed the highest frequency of unique haplotypes, with 26 haplotypes from a total of 33 individuals sampled (Table 1).

The genetic divergence between the haplogroups is 3.88%. The genetic divergence within the population of *P. lucasi* ranged from 0.0% to 4.9% (Table 1), whereas the divergence among population ranged from 0.003% to 0.14% (Table 2). The haplotype diversity (*h*) within the population ranged from 0.73 to 0.99

TABLE 2
Analysis of nucleotide diversity (π), net nucleotide divergence and divergence time estimates (age) among the four populations of *P. lucasi*.

Localities	Distance (KM)	% Pair-wise divergence*†	Nucleotide diversity (π)*†	Net Nucleotide divergence (D_a)	Age of divergence (Kya)#
Miri-Kuching	516.5	0.003	0.01439	-0.00220	7.5
Miri–Sri Aman	420.8	0.13	0.02073	0.02696	325
Miri-Kelantan	1324.4	0.14	0.02061	0.02626	350
Kuching-Sri Aman	210.6	0.14	0.01895	0.02878	350
Kuching-Kelantan	996.9	0.14	0.01877	0.02832	350
Sri Aman-Kelantan	1178.2	0.01	0.00463	0.00327	25

* Estimated using Kimura two-parameter distance (Kimura, 1980).
†Sites with gaps were completely excluded.

TABLE 3
Summary analysis of mtDNA cyt *b* sequences variation among the four populations of *P. lucasi* in Malaysia.

Population	N	H	S	% sdiv	*h*†	π†	K	*D*	F_s	*D**	*F**	r
Miri	33	26	73	0.00 -0.04729	0.985 ± 0.011	0.01584 ± 0.00321	16.80114	-0.29301	**-20.5431***	-0.12175	-0.21304	0.0115
Kuching	33	17	63	0.00 -0.04931	0.938 ± 0.023	0.01316 ± 0.00343	13.96212	-0.37283	**-23.0524***	0.59156	0.31485	0.0220
Sri Aman	6	3	2	0.00 -0.00189	0.733 ± 0.155	0.00082 ± 0.00023	0.86667	-0.05002	**-7.09607***	0.06221	0.03984	0.3467
Kelantan	5	4	12	0.00 -0.00759	0.900 ± 0.161	0.00528 ± 0.00105	5.60000	-0.20090	-1.16655	-0.20090	-0.21293	0.2300
Whole population	77	45	95	0.00 -0.4931	0.978 ± 0.006	0.01964 ± 0.00177	20.83288	0.22450	-6.467	-1.06638	-0.65307	0.0081

N = number of sequence; H = number of haplotypes, S = number of segregating sites, % sdiv = percentage of pair-wise sequence divergence (estimated by K2P distance (Kimura, 1980)); *h* = haplotype diversity; π = nucleotide diversity, K = average number of nucleotide differences; *D* = Tajima's statistics (Tajima 1989), F_s = Fu's statistics (Fu 1997), *D** and *F** = Fu and Li's statistics (Fu & Li, 1993); r = raggedness statistics.
**P* < 0.05, Significance was calculated using coalescent simulation in DnaSP version 4.0 (Rozas *et al.*, 2003).
† Sites with gap were completely excluded.

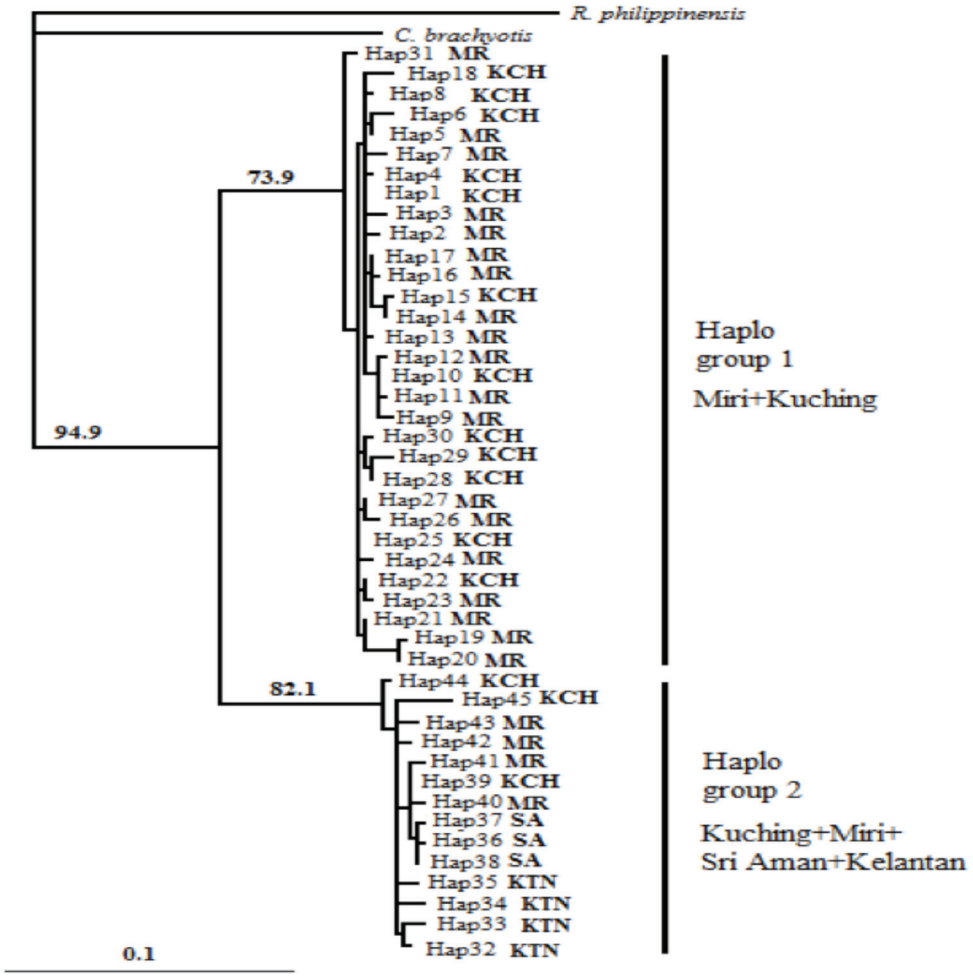

Fig. 2: A maximum likelihood 50% majority rule consensus tree of mtDNA cyt *b* of *P. lucasi*. Bootstrap values above 50 % are indicated below branch. KCH - Kuching; KTN - Kelantan; MR - Miri; SA - Sri Aman.

TABLE 4
Measures of geographical population differentiation in *P. lucasi* based on the analysis of molecular variance (AMOVA)

	Variance component	Percentage % of variation	F-statistic (Φ)	Significant(*P*)
Among groups	9.23414	46.42	Φ_{ct} = 0.46417	0.49970
Among population within groups	3.73415	18.77	Φ_{sc} = 0.35030	0.00000*
Within population	6.92574	34.81	Φ_{st} = 0.65187	0.00000*

*Significant P <0.05

TABLE 5
Genetic differentiation matrix of the populations calculated by Φ_{st} and *P* values is shown in parenthesis.

	Miri	Kuching	Sri Aman	Kelantan
Miri	-			
Kuching	- 0.01525 (0.55856)	-		
Sri Aman	0.65238 (0.0000)*	0.70842 (0.0000)*	-	
Kelantan	0.6335 (0.0000)*	0.69223 (0.0000)*	0.54475 (0.00293)*	-

*Significant $P < 0.05$ with 1000 permutation.

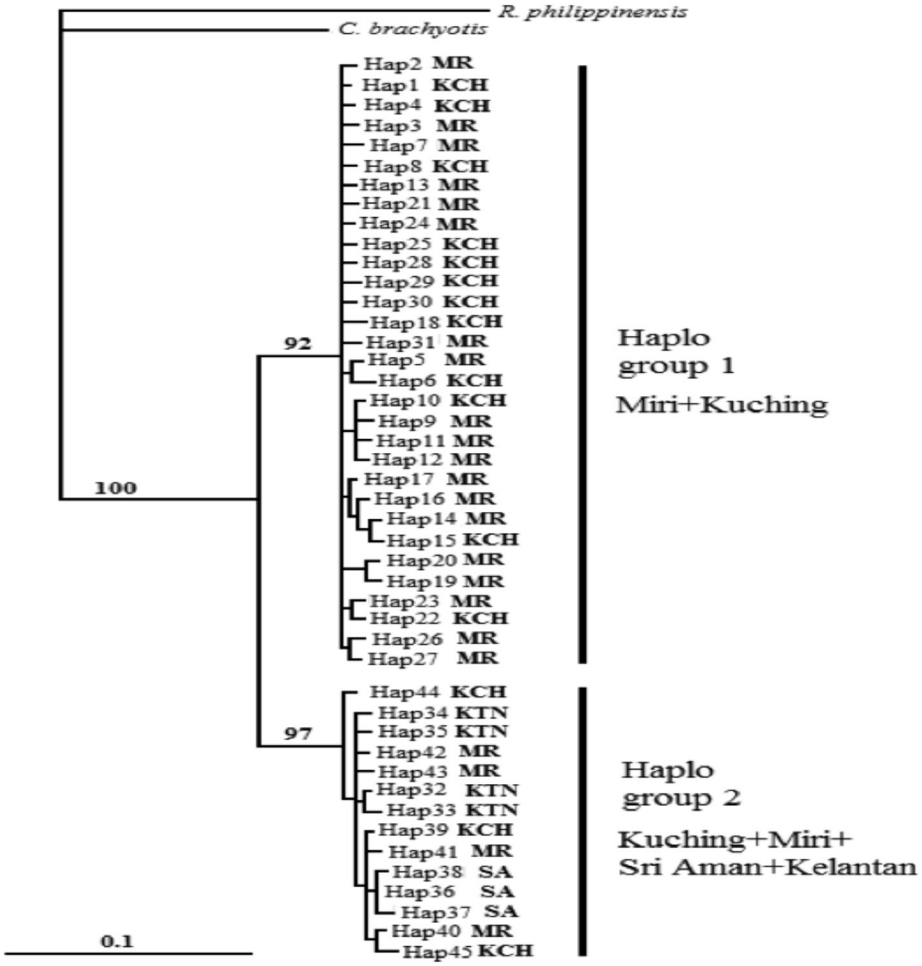

Fig. 3: A Bayesian 50% majority rule consensus tree of mtDNA cyt b of *P. lucasi*. The Bayesian posterior probabilities (BPP) are indicated beside the tree branch nodes: KCH - Kuching; KTN - Kelantan; MR - Miri; SA - Sri Aman.

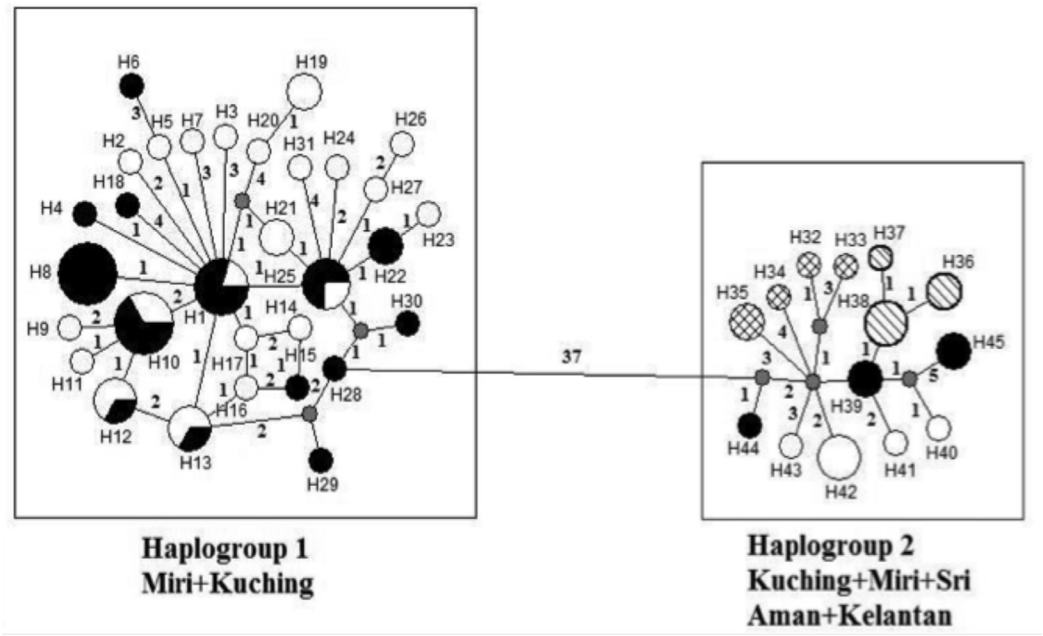

Fig. 4: Haplotype mapping of 45 assigned haplo-nodes within the four populations of *P. lucasi* in Malaysia. All the nodes for the populations of Miri, Kuching, Sri Aman and Kelantan are represented by white, black, forward diagonal and diagonal cross, respectively. The grey nodes represent missing or unsampled haplotypes in this analysis. Note that each node represents unique haplotype and node sizes are proportional to the haplotype frequencies of the given population. Bold numbers indicated at the node branches are the number of mutational steps to connect the nodes. Minimum-spanning network (MSN) was generated by Network 4.5.1.6 program (Fluxus Tech., 2004-2009).

(Table 1). The intra-population nucleotide diversity (π) was high in the Miri population with 0.016.

Among the populations, the nucleotide diversity (π) ranged from 0.004 to 0.02, with an average nucleotide substitutions per site between populations (nucleotide divergence, D_a) ranging from 0.002 to 0.029. A comparison between Miri and Sri Aman showed the highest nucleotide diversity with 0.021 and a divergence (D_a) of 0.027, while the lowest nucleotide diversity of 0.004 was observed between Sri Aman and Kelantan, along with a divergence (D_a) value of 0.003 (Table 2).

The Mantel analysis revealed a lack of significant relationship between nucleotide divergence and geographic distance (correlation coefficient, r = 0.0189, significant P = 0.928) among the four populations of *P. lucasi*. This indicated that the geographical distance was not a contributing factor in the nucleotide divergence within *P. lucasi*.

Neutrality Test and Population Expansion

The neutrality tests of Tajima's D, Fu and Li's, D^* and F^* and Fu's F_s, suggested that there were expansion events within all the *P. lucasi* populations. This was

also supported by a 'star-like' shape of the network of *P. lucasi*. This 'star-like' pattern can be attributed to an expanding population (Slatkin & Hudson, 1991; Rahman, 2000). Tajima's *D* was positive for the total overall population, indicating a lack of recently derived haplotype (Table 3) (Fu & Li, 1993). The negative values of Fu and Li's *D**(-1.06638), Fu and Li's *F**(-0.65307) and Fu's F_s (-6.467) were observed for the total overall population, suggesting the presence of rare haplotypes or polymorphism in the population (Akey *et al.*, 2004; Ramlah, 2009). The analysis for each population also showed a highly significant value of Fu's F_s for the Miri, Kuching and Sri Aman populations (F_s = -20.0525, P = 0.000; F_s = -23.5413, P = 0.000; F_s = -7.0960, P = 0.000, respectively), indicating excess of the recent mutations, while the non-significant value of Fu and Li's *D** and *F** (*D** = -0.1218, P = 0.404; *F** = -0.2130, P = 0.423; *D** = 0.5916, P = 0.7460; *F** = 0.3149, P = 0.678; *D** = 0.0622, P = 0.640; *F** = 0.0398, P = 0.58, respectively) indicated a demographic expansion for each of the populations. However, this was not observed for the Kelantan population.

Population Subdivision

AMOVA was used to determine the extent of population differentiation in *P. lucasi* (Table 4). Population structuring was investigated by grouping the four populations into two broad geographical groups (namely, East and West Malaysia). The grouping was made based on the geographical distance between these two regions within Malaysia which are separated by the South China Sea. A high variation was observed among the groups (46.42%), but was not significantly supported (P = 0.49970). Both the variation among the population within the groups (18.77%) and the variation within (34.81%) the populations were highly significant (P = 0.000). On other hand, the estimated Φ_{st} values among the grouped populations showed a high significance in the pair-wise differentiation (Table 5).

The analysis between the populations revealed high levels of nucleotide (N_{st}) and population subdivision (F_{st}), with low level of migrant per generation (N*m*) between the populations, and the exception between the Miri and Kuching populations. In particular, the *P. lucasi* of both the populations showed a high gene flow (N*m* = 31.72). Despite the closer distance, both the populations in Kuching and Sri Aman showed low levels of migrant per generation (N*m* = 0.30), indicating low female gene flow. Overall, the analyses from the gene flow estimator gave a low level of female migrant per generation of *P. lucasi* in all the populations, except for the population from Miri.

DISCUSSION

Genetic and Population History

Overall, the analysis of 1,061 bp sequences of *P. lucasi* revealed low levels of nucleotide and haplotypes variation. The populations with low level of genetic diversity might have experienced a prolonged or severe demographic bottleneck in the recent times (Avise, 2000). A potential cause for such a bottleneck effect could be due to the

multiple glaciations during Pleistocene epoch (Roques & Negro, 2005; Piaggio *et al.,* 2009). The low levels of genetic variation within *P. lucasi* populations also suggest that they might be recovering from catastrophic or stochastic events during their recent history (Ojeda, 2010). Meanwhile, climatic change and habitat loss may also contribute to reductions in genetic variability of the populations (Hadly *et al.,* 2004; Chan *et al.,* 2005). A study by Chan *et al.* (2005) found that rodent species lost genetic variability as a response to major climatic changes and habitat changes during the Holocene. These conditions may also decrease the population size and range the species (Chan *et al.,* 2005; Roques & Negro, 2005; Piaggio *et al.,* 2009).

Two haplogroups were observed for the *P. lucasi* populations, based on all the haplotype trees and network analyses with a high statistical support, suggesting that the isolation of the haplogroups was not a recent event (Piaggio *et al.,* 2009). A high genetic divergence was found between the two haplogroups (3.88%) in this study. The separation of the haplogroups might be explained in relation to the historical events (Ross *et al.,* 1997; Ramlah, 2009). High mutational steps (37 times) in MSN also suggest that the separation is an ancient event (William *et al.,* 2005). A similar pattern of separation was also found in other taxa, including anurans (Ramlah, 2009) and birds (Ramji, 2010).

Although the historical glacial events appeared to have influenced the genetic structure of the *P. lucasi*, different patterns of colonisation events and refugia could exist between the haplogroups (William *et al.,* 2005; Robert, 2006). The divergence between the haplogroups has a possibility of dating back to 1.95 Mya, which was within the Pleistocene epoch. The mammalian history was typically associated with the Pleistocene event, as it has been known as an important determinant for historical migration. Theoretically, the Sunda Shelf islands, namely, Borneo, Sumatra and Java, had repeatedly merged with Peninsular Malaysia to form a large landmass a number of times (Ruedi & Fumagalli, 1996; Bird *et al.,* 2005). The changing of the sea levels and the fluctuating temperature of the Malay Archipelago during Pleistocene had led to the repeated tropical rain forest isolation and fragmentation, which consequently affected the forest-associated taxa (Ruedi & Fumagalli, 1996; Anthony *et al.,* 2007).

It was hypothesised that some individuals of *P. lucasi* had migrated from their maternal roosts to establish new colonies. These colonies were expected to be surrounded by adequate food resources and secure places for shelter and breeding. As the colonies reached their carrying capacity, the initiator bats were forced to find more fragmented habitats to form new colonies. This stepping stone migration was repeated several times during the Pleistocene climate change period. Eventually, colonies with a common ancestor were assumed to be genetically mixed at intermediate refugia near the water bodies. The northern parts of Borneo (Miri and Sabah) were suggested as the main Quaternary rain forest refugia

in Borneo, as described by many authors (e.g., Ashton, 1972; Brandon-Jones, 1998; Cranbrook, 2000; Morley, 2000; Hunt *et al.,* 2007). The discovery of pollens from Kalimantan also provided the evidence for the existence of the tropical rain forests during LGM (Anshari *et al.,* 2004).

Furthermore, the reduction of moist rainforest, which was concentrated near water bodies, provided refugia for the animals (MacKinnon *et al*., 1996; Morley, 2000). The populations of *P. lucasi* were assumed to be isolated into these refugia over a long period of time. It was further speculated that *P. lucasi* colonised into the tropical rainforest during the interglacial dry period of Pleistocene maximum and dispersed during the cool wet period of Pleistocene minima (Gathorne-Hardy *et al*., 2002), with the spread of the tropical rainforest. Therefore, repeated contraction and expansion of the rainforest during Quaternary would have resulted in two broad haplogroups in the northern and south-western Borneo. It could be hypothesised that such occurrences might have affected the bats in terms of their movement and dispersal abilities. Based on the data obtained in the current study, it could be postulated that the age of divergence for all the populations of *P. lucasi* occurred between 7.5 - 350 kya. The late Pleistocene era dated back to 128 to 11 kya, while the Holocene era began 11 kya and has continued to the present (Cranbrook, 2000). Therefore, part of the divergence events of *P. lucasi* would have occurred from the Holocene to the Late Glacial Maximum (LGM) of Pleistocene epoch.

The placement of haplotypes from Miri and Kuching in both Haplogroup 1 and Haplogroup 2 had led to the occurrence of a species complex which might be present within these populations. A high level of genetic divergence was detected between the haplotypes from all the *P. lucasi* populations (4.9%). Faisal (2008) also found a high divergence of 5% within the populations of *P. lucasi* from Borneo. The author has further suggested that a comprehensive genetic study is needed to verify the divergence. Meanwhile, recent reviews have also suggested that a criterion of 5% sequence divergence in the Cyt *b* gene is considered as an existence of the subspecies, whereas the values exceeding 10% are considered in bats as indicatives of species-level divergence (Bradley & Baker, 2001; Baker & Bradley, 2006). However, the levels of genetic divergence at mtDNA markers alone are not necessarily sufficient to identify the possible cryptic species (Ruedi & McCracken, 2009). Meanwhile, Ibanez *et al.* (2006) proposed species level recognition only to those mtDNA lineages of highly differentiated species (>10%), which also showed morphological differentiation and or ecological isolation. Nonetheless, the assumptions that are solely based on mtDNA markers have been criticised because they reflect only an incomplete part of the natural history of the organisms (Ballard & Whitlock, 2003), or may be misled by the presence of pseudogenes

(Bensasson *et al*., 2001), and/or are affected by the natural limitations of mtDNA markers (Hudson & Turelli, 2003). Due to these possible disadvantages, a cross-validation with independent nuclear markers is highly recommended (Zhang & Hewitt, 2003).

According to Jayaraj (2008), the misclassification of nectarivorous bats into different geographical clades in Malaysia might be due to their ability to perform long-distance flight for foraging. Therefore, this kind of behaviour might explain the misclassification of *P. lucasi* haplotypes from Miri and Kuching present in both haplogroups. The Old World fruit bats can travel up to hundreds of kilometres, both within the mainland and across the ocean barriers (Shilton *et al.,* 1999). Some good examples of the local species are *Eonycteris spelaea* and *C. brachyotis*, which can travel up to 50 km for foraging in a single night (Fukuda *et al.,* 2009). The high mobility of these species has made them very successful in terms of distribution; they can be found to inhabit various types of vegetations, from the lowland dipterocarp forest, peat swamp forest, kerangas, and up to montane forest (Payne *et al.,* 1985; Francis, 2008). As a megabat, *P. lucasi* is capable of travelling long distances and foraging in more places. This enables individuals to migrate from the north to the south of Sarawak, or *vice versa*. This is further demonstrated by the colonisation of bats in Krakatau Island, which proves that the bodies of water or oceans are not an effective barrier to impede the dispersion of the species of fruit bats (Whittaker & Jones, 1994; Thornton *et al.,* 1996).

Population Partitioning and Gene Flow

Gene flow

The level of gene flow is expected to decrease with the increase of distance between two or more populations (Karuppudurai *et al*., 2007). Consequently, the nearest population is more similar at the neutral loci (Storz, 2002). This relationship refers to the isolation by distance, and assumes a stepping stone model of gene flow, which will provide a sufficient time for the population to reach a condition of equilibrium (Kimura & Weiss, 1964). However, the levels of gene flow are not only dependent on the distance between the populations, but also on the environment of the surrounding landscape between the populations (Storz, 2002). Thus, a high level of genetic variation within a population could result in a high level of gene flow, specifically for the populations in Miri and Kuching (Karuppudurai *et al*., 2007). This can be assumed since the sharing of haplotypes has been observed only (between) in the populations in Miri and Kuching, despite their notable distance from each other. This could have resulted from the continuous distribution of the *P. lucasi* population.

In sedentary species, extrinsic barriers to gene flow and historical events may determine the extent of genetic partitioning among the populations (Karuppudurai *et al.,* 2007). A barrier such as a developed area separating these localities has been suggested as a factor contributing to the failure of this particular species to be connected with each other and hence, impedes any gene flow between the populations (Storz, 2002).

Fluctuations in the world's temperature and a series of lowering and rising of sea levels during the late Pleistocene might have somehow affected this particular species since it depended on the forest for food. These phenomena have also allowed for the formation of different types of forest (Campbell *et al.,* 2006). According to Hudson *et al*. (1992), a significant differentiation between the populations would be expected only if the N*m* value was < 1.0. Similar results have been reported in *P. poliochepalus* and *P. alecto* (Webb & Tideman, 1996); *Plecotus auritus* (Burland *et al.,* 1999); *M. lyra* (Rajan & Marimuthu, 2006) and *C. sphinx* (Karuppudurai, 2007). As for the populations of *P. lucasi*, only one population interaction showed a deviated value with its N*m*>1, i.e. the Miri-Kuching populations. The non-significant correlation between the geographical distance and the genetic diversity among the populations of *P. lucasi* in Malaysia has led to the rejection of genetic isolation by geographic distance. Therefore, factors other than the distance between the populations are responsible for the differentiation observed in the populations of *P. lucasi*.

CONCLUSION

The findings of the current study indicated that the age of divergence for all the populations of *P. lucasi* occurred between 350 – 7.5 kya. The divergence within the populations in Miri (4.9%) and Kuching (4.7%) could have led to the occurrence of a species complex within *P. lucasi*. The presence of the haplotypes from both the populations in Haplogroup 1 and Haplogroup 2 is due to the ability of the dusky fruit bats to perform long-distance flights for foraging. A high gene flow was detected between these populations, suggesting continuous "stepping-stone" distributions of *P. lucasi,* despite the existing considerable distance between both localities. Meanwhile, the absence of a deep structure from the haplotype trees suggested that *P. lucasi* has a wide dispersal ability. The populations of *P. lucasi* were also expected to experience interpopulation genetic divergence, which could be classified into different evolutionary significant units (ESU) for management purposes. This study provided some useful insights into the phylogeoraphic relationships, genetic uniqueness, and population structure of *P. lucasi* in Malaysia. However, further studies should be carried out using larger sample sizes per population and samples from other cave areas (e.g. Mulu in Sarawak, Gomantong and Madai in Sabah) within their geographical distribution for conservation management strategies of the populations of *P. lucasi,* which are highly dependent on the cave system for breeding and shelter, and the surrounding forested areas for food resources. Additionally, information based on the nuclear DNA markers and fast evolving mtDNA genes (microsatellites) is necessary to elucidate the complex status of *P. lucasi*.

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APPENDIX 1

List of samples of *P. lucasi* used in the genetic analyses.

No	Species	Voucher/ Museum. No	Locality	Habitat	GenBank Acc. No.
1	*P. lucasi*	MZU/M/02120	Niah NP, Miri, Sarawak	Limestone forest	GU724886
2	*P. lucasi*	MZU/M/02122	Niah NP, Miri, Sarawak	Limestone forest	GU724906
3	*P. lucasi*	MZU/M/02123	Niah NP, Miri, Sarawak	Limestone forest	GU724887
4	*P. lucasi*	MZU/M/02124	Niah NP, Miri, Sarawak	Limestone forest	GU724932
5	*P. lucasi*	MZU/M/02125	Niah NP, Miri, Sarawak	Limestone forest	GU724933
6	*P. lucasi*	MZU/M/02127	Niah NP, Miri, Sarawak	Limestone forest	GU724888
7	*P. lucasi*	MZU/M/02128	Niah NP, Miri, Sarawak	Limestone forest	GU724889
8	*P. lucasi*	MZU/M/02130	Niah NP, Miri, Sarawak	Limestone forest	GU724890
9	*P. lucasi*	MZU/M/02131	Niah NP, Miri, Sarawak	Limestone forest	GU724891
10	*P. lucasi*	MZU/M/02133	Niah NP, Miri, Sarawak	Limestone forest	GU724934
11	*P. lucasi*	MZU/M/02134	Niah NP, Miri, Sarawak	Limestone forest	GU724892
12	*P. lucasi*	MZU/M/02135	Niah NP, Miri, Sarawak	Limestone forest	GU724907
13	*P. lucasi*	MZU/M/02153	Niah NP, Miri, Sarawak	Limestone forest	GU724935
14	*P. lucasi*	MZU/M/02154	Niah NP, Miri, Sarawak	Limestone forest	GU724908
15	*P. lucasi*	MZU/M/02155	Niah NP, Miri, Sarawak	Limestone forest	GU724936
16	*P. lucasi*	MZU/M/02156	Niah NP, Miri, Sarawak	Limestone forest	GU724937
17	*P. lucasi*	MZU/M/02157	Niah NP, Miri, Sarawak	Limestone forest	GU724893
18	*P. lucasi*	MZU/M/02163	Niah NP, Miri, Sarawak	Limestone forest	GU724909
19	*P. lucasi*	MZU/M/02169	Niah NP, Miri, Sarawak	Limestone forest	GU724894
20	*P. lucasi*	TK152463	Niah NP, Miri, Sarawak	Limestone forest	GU724895
21	*P. lucasi*	TK152468	Niah NP, Miri, Sarawak	Limestone forest	GU724896
22	*P. lucasi*	TK152470	Niah NP, Miri, Sarawak	Limestone forest	GU724897
23	*P. lucasi*	TK152481	Niah NP, Miri, Sarawak	Limestone forest	GU724910
24	*P. lucasi*	TK152482	Niah NP, Miri, Sarawak	Limestone forest	GU724929
25	*P. lucasi*	TK152483	Niah NP, Miri, Sarawak	Limestone forest	GU724911
26	*P. lucasi*	TK152933	Niah NP, Miri, Sarawak	Limestone forest	GU724898
27	*P. lucasi*	TK152953	Niah NP, Miri, Sarawak	Limestone forest	GU724899
28	*P. lucasi*	TK152954	Niah NP, Miri, Sarawak	Limestone forest	GU724900
29	*P. lucasi*	TK152964	Niah NP, Miri, Sarawak	Limestone forest	GU724912
30	*P. lucasi*	TK152965	Niah NP, Miri, Sarawak	Limestone forest	GU724901
31	*P. lucasi*	TK152966	Niah NP, Miri, Sarawak	Limestone forest	GU724902
32	*P. lucasi*	TK152971	Niah NP, Miri, Sarawak	Limestone forest	GU724930

33	*P. lucasi*	MZU/M/01685	Lambir NP, Miri, Sarawak	Lowland Dipterocarp Forest	GU724954
34	*P. lucasi*	TK152883	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724938
35	*P. lucasi*	TK152884	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724939
36	*P. lucasi*	TK152885	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724940
37	*P. lucasi*	TK152887	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724941
38	*P. lucasi*	MZU/M/02173	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724942
39	*P. lucasi*	MZU/M/02180	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724943
40	*P. lucasi*	MZU/M/02207	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724904
41	*P. lucasi*	MZU/M/02209	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724914
42	*P. lucasi*	MZU/M/02210	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724905
43	*P. lucasi*	MZU/M/02211	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724915
44	*P. lucasi*	MZU/M/02212	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724916
45	*P. lucasi*	MZU/M/02214	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724917
46	*P. lucasi*	MZU/M/02216	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724918
47	*P. lucasi*	MZU/M/02217	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724919
48	*P. lucasi*	MZU/M/02226	Wind Cave NR, Kuching, Sarawak	Secondary forest	GU724920
49	*P. lucasi*	MZU/M/02227	Wind Cave NR, Kuching, Sarawak	Secondary forest	GU724921
50	*P. lucasi*	MZU/M/02232	Wind Cave NR, Kuching, Sarawak	Secondary forest	GU724922
51	*P. lucasi*	MZU/M/02229	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724923
52	*P. lucasi*	MZU/M/02233	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724927

53	*P. lucasi*	MZU/M/02235	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724925
54	*P. lucasi*	MZU/M/02236	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724926
55	*P. lucasi*	MZU/M/02234	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724924
56	*P. lucasi*	MZU/M/02238	Wind Cave NR, Kuching, Sarawak	Limestone forest	GU724928
57	*P. lucasi*	MZU/M/01716	Kubah NP, Kuching, Sarawak	Mixed Dipterocarp Forest	GU724903
58	*P. lucasi*	MZU/M/02239	Padawan, Kuching, Sarawak	Limestone forest	GU724953
59	*P. lucasi*	MZU/M/02240	Padawan, Kuching, Sarawak	Limestone forest	GU724885
60	*P. lucasi*	MZU/M/02241	Padawan, Kuching, Sarawak	Limestone forest	GU724931
61	*P. lucasi*	MZU/M/00568	Mount Penrissen, Kuching, Sarawak	Montane forest	GU724952
62	*P. lucasi*	MZU/M/00569	Mount Penrissen, Kuching, Sarawak	Montane forest	GU724913
63	*P. lucasi*	MZU/M/00570	Mount Penrissen, Kuching, Sarawak	Montane forest	GU724950
64	*P. lucasi*	MZU/M/02242	Bako NP, Kuching, Sarawak	Mixed Dipterocarp Forest	GU724948
65	*P. lucasi*	MZU/M/02243	Bako NP, Kuching, Sarawak	Mixed Dipterocarp Forest	GU724955
66	*P. lucasi*	MZU/M/02244	Bako NP, Kuching, Sarawak	Mixed Dipterocarp Forest	GU724949
67	*P. lucasi*	MZU/M/01192	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724881
68	*P. lucasi*	MZU/M/01193	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724882
69	*P. lucasi*	MZU/M/01190	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724951
70	*P. lucasi*	MZU/M/01194	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724883

71	*P. lucasi*	MZU/M/01191	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724947
72	*P. lucasi*	MZU/M/01195	Batang Ai NP, Sri Aman, Sarawak	Lowland Dipterocarp Forest	GU724884
73	*P. lucasi*	DWNP 02142	Gua Musang, Kelantan	NA	GU724879
74	*P. lucasi*	DWNP 02143	Gua Musang, Kelantan	NA	GU724945
75	*P. lucasi*	DWNP 02144	Gua Musang, Kelantan	NA	GU724880
76	*P. lucasi*	DWNP 02145	Gua Musang, Kelantan	NA	GU724946
77	*P. lucasi*	DWNP 02375	Gua Musang, Kelantan	NA	GU724944
78	*C.brachyotis*	TK152458	Mount Murud, Miri, Sarawak	Montane forest	GU724956
79	*R. philippinensis*	TK152938	Niah NP, Miri, Sarawak	Limestone forest	GU724957

NA= Not available; NP= National Park; NR= Nature Reserve.

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Phylogeny and Phylogeography of *Aethalops* from Sundaland using Mitochondrial 12S rRNA Gene

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ABSTRACT

One of the smallest fruit bats in Pteropodidae is *Aethalops*. This genus is known to be confined in montane forest, which is generally above 1000 meters above sea level (m.a.s.l.). Bornean *Aethalops* is generally known as *Aethalops alecto* in most previous literature. This study aimed at constructing the phylogenetic relationship of *A. alecto* and *A. aequalis* in Sundaland and determining gene flow within Bornean *A. aequalis* using partial mitochondrial 12S rRNA gene. Seven populations of *A. aequalis,* representing Sabah and Sarawak and a single population from Kalimantan were observed, whereas *A. alecto* were represented by four populations from Indonesian islands. From the phylogenetic analyses and minimum spanning network, there were two major clusters within the genus, with *Aethalops*. *A. aequalis* in Borneo were clearly distinguished from *A. alecto* from the islands of Indonesia. However, phylogenetic analyses within *A. aequalis* were unresolved at the population levels in Sabah and Sarawak. Therefore, it can be concluded that *A. aequalis* is the species found only in Borneo. High genetic similarities were detected among the populations of *A. aequalis* in Sabah and Sarawak. Hypothetically, the Kalimantan harbors ancestral populations of *A. aequalis* in Borneo, with high genetic divergence from Sabah and Sarawak populations.

Keywords: Aethalops, populations, phylogeny, phylogeography, Sundaland, 12S rRNA

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INTRODUCTION

The montane bat *Aethalops* is among of the smallest Old World fruit bat (Pteropodidae), which is also known as Pigmy Fruit Bat or tailless fruit bat. *Aethalops* is confined in the montane forest above 1000 m (Payne *et*

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al., 1985; Mickleburgh *et al*., 1992; Francis, 2005) and has a low widespread area (Kitchener *et al*., 1993). The fur is grey-brown to reddish brown, and thick and long on the dorsal surface. The muzzle is narrow and pointed and forearm length is between 42 – 46 mm (Payne *et al*., 1985). The distinctive characteristics that differentiate this genus from its sister genus, *Balionycteris* (Ryan *et al*., 2008) is that the *Aethalops* are tailless, spotless on the wings and have a pair of lower incisors (Payne *et al*., 1985). *Aethalops* are found throughout Peninsular Malaysia, Sundaland, and other islands in Indonesia. Sundaland refers to Sumatra, Java, Lombok, Borneo and Peninsular Malaysia. There are two species within the genus, namely *A. alecto* and *A. aequalis*, and both are endemic to the mountainous areas. Previous studies have indicated that there is a distribution boundary between the two species (Kitchener *et al*., 1993; Maharadatunkamsi *et al*., 2006). However, some authors still consider *Aethalops* in Borneo as *A. alecto* rather than *A. aequalis* (Payne *et al*., 1985; Francis, 2005). Bornean *Aethalops* or Bornean Pigmy Fruit Bats (*A. a. aequalis*) are considered as a sub-species

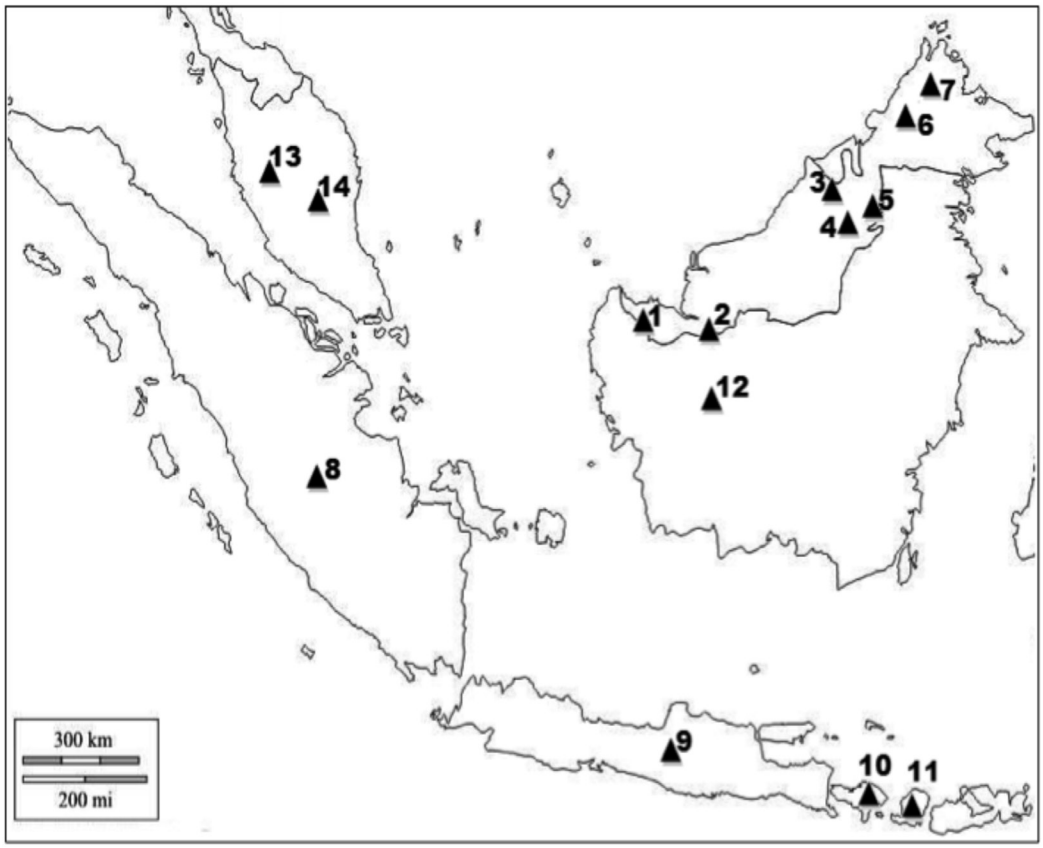

Fig. 1: Distributions of *A. aequalis* based from the specimens used in this study (1-12) and the sampling sites for *A. alecto* (13-14). 1-Mt Pueh; 2-Mt Penrissen; 3-Mt Mulu; 4-Bario; 5-Mt Murud; 6-Mt Trus Madi; 7- Mt Kinabalu; 8-Sumatra; 9- Java; 10-Bali; 11-Lombok; 12- Kalimantan; 13-Fraser's Hill; 14-Mt Benom

of *A. alecto* (Hill, 1961, 1966; Hill, 1983; Boeadi & Hill, 1986; van Strien, 1986; Koopman, 1989).

In this paper, the phylogenetic relationships of Sundaland *Aethalops* were assessed using 12S rRNA on the *Aethalops* of Sundaland. The aims of this study were to construct the phylogenetic relationship of *A. alecto* and *A. aequalis* and determine the patterns of gene flow of *A. aequalis* within Borneo, as evident in the partial mitochondrial 12S rRNA gene.

TABLE 1
List of the specimens, museum reference, location, abbreviation (Abbr.) and GenBank accesion numbers

Locality	Elevation	FA	Sex	Abbr.	Field No.	UNIMAS Voucher No.	Accession No.
Mt Kinabalu	1527 m a.s.l	44.50	M	TK4	TK004	UNIMAS 00371	HM067793
		42.18	F	TK20	TK152920	-	HM067816
		44.31	F	TK21	TK152921	-	HM067774
		42.94	M	TK22	TK152922	-	HM067775
		44.25	F	TK23	TK152923	-	HM067814
		43.92	M	TK24	TK152924	-	HM067776
		42.54	F	TK25	TK152925	-	HM067777
		46.98	F	TK26	TK152926	-	HM067778
		45.53	M	TK27	TK152927	-	HM067779
		43.82	F	TK28	TK152928	-	HM067780
		44.29	F	TK30	TK152930	-	HM067815
Mt Trus Madi	1446 m a.s.l	43.16	F	TM1	TM001	-	HM067781
		44.17	F	TM2	TM011	-	HM067785
		44.59	F	TM3	TM012	-	HM067782
		43.59	F	TM4	TM013	-	HM067786
		41.53	F	TM5	TM014	-	HM067801
		43.77	F	TM6	TM015	-	HM067783
Mt Murud	1335-2113 m a.s.l	44.35	M	MRD1	RV018	UNIMAS 01015	HM067760
		32.84	F	MRD2	RV042	UNIMAS 01016	HM067804
		43.73	M	MRD3	MRT004	UNIMAS 01127	HM067787
		45.1	M	MRD5	MRT010	UNIMAS 01129	HM067788
		44.22	F	MRD7	RV 019	UNIMAS 01361	HM067802
		40.25	F	MRD9	RV 032	UNIMAS 01363	HM067803
		45.46	F	MRD10	RV 041	UNIMAS 01364	HM067798
		43.68	F	MRD11	RV 027	UNIMAS 01365	HM067762
		41.86	F	MRD12	RV 013	UNIMAS 01366	HM067807

Table 1 (*continued*)

Locality	Elevation	FA (mm)	Sex	Abbr.	Field No.	UNIMAS Voucher No.	Accession No
Mt Murud	1335-2113 m a.s.l	45.82	F	MRD13	RV 008	UNIMAS 01367	HM067805
		41.48	F	MRD14	RV 012	UNIMAS 01368	HM067789
		42.16	F	MRD15	RV 011	UNIMAS 01369	HM067811
		46.18	F	MRD16	RV 027	UNIMAS 01370	HM067817
		41.48	F	MRD17	RV 029	UNIMAS 01371	HM067790
		43.27	F	MRD18	RV 005	UNIMAS 01372	HM067791
		43.27	F	MRD19	RV 010	UNIMAS 01373	HM067758
		44.39	F	MRD20	RV 006	UNIMAS 01374	HM067812
		45.34	M	MRD21	RV 007	UNIMAS 01375	HM067808
		45.12	F	MRD22	RV 009	UNIMAS 01376	HM067809
		44.9	M	MRD23	Mrd004	-	HM067763
		41.96	F	MRD24	Mrd007	-	HM067764
		43.64	F	MRD25	Mrd008	-	HM067765
		42.38	F	MRD26	Mrd009	-	HM067766
		42.77	M	MRD27	Mrd015	-	HM067767
Mt Mulu	1764 m a.s.l	45.40	F	MU1	Berta1	-	HM067768
		42.23	F	MU2	Berta2	-	HM067769
		42.55	F	MU3	Berta3	-	HM067800
		42.26	F	MU4	Berta4	-	HM067770
		41.63	F	MU5	Berta5	-	HM067771
		44.22	M	MU6	Berta6	-	HM067772
		42.67	M	MU7	MMB3	-	HM067795
		43.69	M	MU8	MMB4	-	HM067796
		43.45	F	MU9	MMB5	-	HM067797
Bario	1100-1250 m a.s.l	44	M	Bar3	BD016	UNIMAS 00053	HM067810
Mt Penrissen	746-1000 m a.s.l	45.7	M	MP2	MP03	UNIMAS 00590	HM067794
		43.09	M	MP3	MP06	UNIMAS 00591	HM067761

Table 1 (*continued*)

Locality	Elevation	FA	Sex	Abbr.	Field No.	UNIMAS Voucher No.	Accession No
Mt Penrissen	746-1000 m a.s.l	43.75	F	MP4	MP001	-	HM067759
		-	F	MP5	MP016	-	HM067813
		44.94	F	MP6	MP020	-	HM067784
		42.03	M	BOH1	PB 035	UNIMAS 00678	HM067806
				BOH2		UNIMAS 00679	HM067792
		44.62	M	BOH3	BH 76	UNIMAS 01525	HM067799
Mt Pueh	845m a.s.l	-	F	PUEH1	1046	UNIMAS 01632	HM067773

MATERIALS AND METHODS

Samples were collected from nine sites, namely, Southwest Sarawak group [Mount (Mt) Penrissen and Mt Pueh], Northeast Sarawak group (Mt Murud, Mt Mulu and Bario) and Sabah group (Mt Kinabalu and Mt Trus Madi) and Peninsular Malaysia (Fraser's Hill and Mount Benom) (Table 1 and Fig.1). Mist nets were set along the forest trail, near streams and on the forest edge. Captured bats were identified and measured following Payne *et al*. (1985) and deposited in the Zoological Museum of Universiti Malaysia Sarawak (Abdullah *et al*., 2010). Selected bats were preserved either as wet or dry specimens, and the others were released with marked bands. Tissue samples were taken from the pectoral part of the body and preserved either in lysis buffer or ethanol.

Total genomic DNA of *A. aequalis* was then extracted using the modified 2X cetil – trimethylammonium bromide (CTAB) method, following Grewe *et al*. (1993). Partial mitochondrial 12S rRNA gene was amplified with primer 12SA-L 5' – aaa ctg gga tta gat acc cca – 3' and and 12SA-H 5' – atg ttt ttg ata aac agg – 3' (Palumbi *et al*., 1991). The template DNA was amplified in 25 µl of the reaction mixture containing 5 µl of 5x buffer (Promega), 1.5 µl of 25 mM $MgCl_2$ (Promega), 0.2 µl of dNTP (10 mM) (Promega), 0.1 µl of each primer (10 mM) and 0.2 units of *Taq* polymerase (Promega). The cycle parameters consisted of 30 cycles of denaturation (at 94°C for 1 minute), annealing (55 – 58°C for 1 minute) and extension (at 72°C for 2 minutes). The amplified products were visualised on 2% agarose gels containing ethidium bromide, run on gel electrophoresis for 30 minutes at 90V, and photographed under the ultraviolet light. GeneRuler™ 100 bp DNA ladder was used as a standard size marker (Promega). Purified products were sent to private laboratories for sequencing using ABI prism ™ Big dye ™ terminator cycle sequencing Ready kit version 3.1, or using the ABI PRISM® 377 DNA Sequencer with the BigDye® Terminator v3.0 Cycle Sequencing Kit and the sequencing product was run using ABI 3730 XL capillary DNA sequencer (50 cm capillary).

TABLE 2
List of the 12S rRNA sequences of *A. aequalis* and *A. alecto* from Indonesia (obtained from the GenBank, as well as the longitude and latitude estimated from Google Map)

Species	Locality	Accession number (Field no.)	Longitude/ Latitude	Elevation (m) a.s.l
A. alecto	Gunung Rinjani, Lombok	DQ845089 (GR1)	116° 28'00"E 08° 25'00"S	3726
	Gunung Rinjani, Lombok	DQ845088 (GR2)	116° 28'00"E 08° 25'00"S	3726
	Batang Toru, Sumatra	DQ845091 (BT1)	98° 53' – 99° 26'E 02° 03' – 01° 27'N	400 - 1803
	Kebun Raya Eka Karya, Bali	DQ845086 (KREK1)	115° 22' 30"E 8° 14' 30"S	1400
	Taman Nasional Gunung Halimunan, Java	DQ845081 (TNGH1)	106° 21' – 106° 31'E 06° 37' – 06° 51'S	500 - 1929
	Taman Nasional Gunung Halimunan, Java	DQ845080 (TNGH2)	106° 21' – 106° 31'E 06° 37' – 06° 51'S	500 - 1929
	Kebun Raya Cibodas, Java	DQ845082 (KRC5)	107° 37' 53.4"E 06° 51' 53.2794"S	1250
	Kebun Raya Cibodas, Java	DQ845083 (KRC6)	107° 37' 53.4"E 06° 51' 53.2794"S	1250
	Kebun Raya Cibodas, Java	DQ845084 (KRC2)	107° 37' 53.4"E 06° 51' 53.2794"S	1250
	Tahura Raden Soeryo, Java	DQ845085 (TRS3)	31°'44.69"E 7° 44'12.58"S 112	2227
	Tahura Raden Soeryo, Java	DQ845087 (TRS4)	31°'44.69"E 7° 44'12.58"S 112	2227
	Tahura Raden Soeryo, Java	DQ845090 (TRS2)	31°'44.69"E 7° 44'12.58"S 112	2227
A.aequalis	Taman Nasional Bukit Baka, Kalimantan	DQ845096 (BBBR1)	112° 50'E 0° 47'S	150 - 2278
	Taman Nasional Bukit Baka, Kalimantan	DQ845092 (BBBR2)	112° 50'E 0° 47'S	150 - 2278
	Taman Nasional Bukit Baka, Kalimantan	DQ845093 (BBBR3)	112° 50'E 0° 47'S	150 - 2278
	Taman Nasional Bukit Baka, Kalimantan	DQ845094 (BBBR4)	112° 50'E 0° 47'S	150 - 2278
	Taman Nasional Bukit Baka, Kalimantan	DQ845095 (BBBR5)	112° 50'E 0° 47'S	150 - 2278

The fluorescence-based DNA sequences were displayed using Chromas version 1.45 (McCarthy, 1996). CLUSTAL X version 1.8 (Thompson *et al.,* 1997) was used to align the DNA sequences. After the alignment, the DNA sequences were blasted in NCBI Blast for species confirmation. Additional sequences of 12S rRNA were obtained from GenBank (Table 2). Pair-wise distance between the populations was performed in Molecular Evolutionary Genetic Analysis (MEGA) 4.0 using Kimura-2-parameter (K2P) model (Kimura, 1980). Evolutionary model for 12S rRNA gene was conducted from Modeltest 3.7, and the best model was selected by Akaike Information Criterion (AIC) (Pasoda & Crandall, 1998). Phylogenetic trees was constructed using Neighbour Joining (NJ), while Maximum Parsimony (MP) and Maximum Likelihood (ML) were implemented in Phylogenetic Analysis Using Parsimony (PAUP version 4.0 beta; Swofford, 1998), and the Bayesian tree was constructed in MrBayes (Huelsenbeck & Rosquist, 2001). The Bootstrap method with NJ search (Saitou & Nei, 1987) was conducted using PAUP version 4.0 beta with 1000 replicates. For character-based method, the MP and ML methods were applied to estimate the phylogenetic relationship study for discrete data. Meanwhile, the Heuristic searches for MP analysis were performed with 10 random additions of taxa. The reliability of the nodes defined by the phylogenetic trees was assessed using 1000 bootstrap iterations in the fast heuristics modes.

The ML analysis was performed based from the best fit evolutionary model selected by AIC. The Heuristic search option was used in PAUP* with tree-bisection-reconnection (TBR) branch swapping and 10 random addition sequence replicates. Tree-bisection-reconnection (TBR) was used as the branch-swapping algorithm. The consensus tree from a parsimony heuristic search was used to evaluate the ML tree.

The Bayesian analysis (MrBayes 3.1.2, Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) was performed with 100^{th} generations implementing Metropolis-coupled Markov chain Monte Carlo (MCMC) under best selected model by AIC, each with four independent incrementally heated Markov chains, sampling every 100^{th} generation and burn-in of 1000 for summary parameter values and trees. The convergence of the two runs was assumed when the average standard deviation of the split frequencies has reached less than 0.1 and the potential scale reduction factor approached 1.00.

Haplotype and nucleotide diversity, Pi (p) (Nei, 1987) were calculated in DnaSP (Rozas *et al*., 2003) by using Nei's (1987) indices. The nucleotides divergence among the populations was estimated in DnaSP (Rozas *et al*., 2003). Meanwhile, the number of haplotype, segregating sites and total number of mutations were estimated using DnaSP (Rozas *et al*., 2003).

Genetic differentiation (F_{st}, N_{st} and N*m* values) was implemented in DnaSP (Rozas *et al*., 2003), whereas a hierarchical

analysis (Analysis of Molecular Variance or AMOVA), and Mantel test were estimated using Arlequin software (Excoffier, 2005).

The significance level of the F_{st} values was determined by a permutating test between the localities ($p < 0.05$). F_{st}, which is the population subdivision index, was calculated to describe the reduction in heterozigosity relative to the total population which are due to selection or drift. In fact, F_{st} is the most common measurement used to describe the genetic differentiation of the populations and was developed by Wright (1951). F_{st} is the value of probability of two random gametes which were drawn from two populations that are identical by descent, and relative to gametes taken from the entire populations. The F_{st} values ranging from of 0.00 – 0.05 are commonly considered as having little genetic differentiation, whereas 0.05 – 0.25 commonly indicates moderate genetic differentiation, and the values > 0.25 signify a pronounced level of genetic differentiation (Lowe *et al*., 2004).

N_{st} is used to estimate the degree of populations' subdivision at the nucleotide level, with the values ranging from 0 (no population subdivision) to 1 (complete population subdivision) (Bouga *et al*., 2005), of which, it describes the genetic differentiation within the species (Riginos *et al*., 2010).

The Mantel test was conducted in Arlequin (Excoffier, 2005) to estimate isolation by distance. A statistical method that uses permutations to test the null hypothesis, i.e. two variables were independent of each other and a statistical approach, was used to compare the geographical distance and genetic differentiation among the populations; in other words, to test for the isolation by distance. The significance level was tested using 1000 permutations.

Gene flow, N*m* i.e. the number of migrants per generation, was also implemented in DnaSP (Rozas *et al*., 2003). When the value of Nm is less than 1 ($F_{st} = 0.2$), the population is expected to genetically diverge over time. However, if N*m* is more than 1, the populations are expected to retain genetic connectivity.

The 12S rRNA gene constant transversion rate for mammals (bats) was estimated following Mindell *et al*. (1991), which is 0.27%. Formula divergence time (Rustchmann, 2006) is T = % net mean PD / 2r (T = time of divergence; PD = pair-wise distance; r = constant transversion rate).

RESULTS

Fig.1 shows the sampling sites of *A. aequalis* and *A. alecto* used in this study. However, *A. alecto* was unsuccessfully to be captured at Fraser's Hill and Mt Benom. From 72 individuals, including two outroups, 69 were successfully sequenced and aligned for a total of 290 bp of 12S rRNA gene. The highest nucleotide frequencies in 12S rRNA of genus *Aethalops* were adenine (A), with the average value of 38.0%, followed by thymine (T) with 21.6%, cytosine (C) with 20.6% and guanine (G) with 19.8%. The nucleotide composition showed an anti-G bias with the least frequencies of C and G (40.5%), as compared to A and T (59.5%), a

TABLE 3
Above diagonal is the mean pairwise distance within (bold) and between the populations of *A. aequalis*. The maximum and minimum ranges of the pair-wise distance are shown in parenthesis. Below diagonal is the geographical distance between localities

	Kinabalu	Trus Madi	Murud	Mulu	Bario	Penrissen	Pueh	Kalimantan
Kinabalu	**0.68 (0.0-3.6)**	0.35 (0.0-2.1)	0.49 (0.0-2.8)	0.52 (0.0-2.8)	3.9 (3.5-5.8)	0.79 (0.0-2.1)	0.35 (0.0-2.1)	4.79 (3.5-6.1)
Trus Madi	58.3	**0 (0.0)**	0.12 (0.0-0.7)	0.23 (0.0-0.7)	3.5 (3.5)	0.43 (0.0-0.7)	0.0 (0.0)	4.49 (3.5-5.0)
Murud	268	224	**0.32 (0.0-1.4)**	0.34 (0.0-1.4)	3.72 (3.5-4.3)	0.59 (0.0-1.4)	0.17 (0.0-0.7)	4.61 (3.5-5.4)
Mulu	279	242	46.8	**0.38 (0.0-0.7)**	3.5-4.3	0.67 (0.0-1.4)	0.23 (0.0-0.7)	4.65 (3.5-5.4)
Bario	284	238	30.5	79.1	**0.0**	3.99 (3.5-4.3)	3.5 (3.5)	8.25 (7.3-8.8)
Penrissen	868	830	626	591	629	**0.35 (0.0-0.7)**	0.43 (0.0-0.7)	4.95 (3.5-5.7)
Pueh	901	865	670	636	676	85.9	**0.0 (0.0)**	4.49 (3.5-4.6)
Kalimantan	845.4	791.4	576.6	560.4	541.3	344.7	414.2	**1.61 (0.0-2.1)**

TABLE 4
List of the haplotypes found in each population in Sundaland

	Kinabalu (12)	Trus Madi(6)	Murud (24)	Mulu (9)	Bario (1)	Penrissen (8)	Pueh (1)	Kalimantan (5)	Lombok (2)	Bali (1)	Sumatra (1)	Java (8)
Aa1									1			
Aa2									1			
Aa3										1		2
Aa4												1
Aa5												1
Aa6												3
Aa7												1
Aa8											1	
Aae1								1				
Aae2								1				
Aae3								1				
Aae4								2				
Aae5						4						
Aae6			1			2						
Aae7				1								
Aae8	9	6	17	5		2	1					
Aae9			1									
Aae10			1									
Aae11					1							
Aae12	1											
Aae13	1		4	2								
Aae14	1											
Aae15				1								

*Figures in parenthesis indicate the total number of individuals from each population.
Shaded represents the haplotype for *A. alecto*.

characteristic indicating mitochondrial gene. From 290 bp, 219 (84.5%) were conserved sites and 71 (14.5%) were variable sites, with 33 parsimoniously informative sites.

The genetic distance of *A. aequalis* populations within Malaysian Borneo ranged from 0.0 – 5.8%, and this was 0.0 – 2.1% (mean divergence = 1.6%) within the populations in Kalimantan. Within the populations in Sabah, it encounters 0.0 – 3.6% (mean divergence = 0.5%), and this ranged from 0.0 – 4.3% (mean divergence = 0.6%) of genetic divergence for the populations in Sarawak. Overall, the genetic distance among the species *A. aequalis* ranging from 0.0 to 8.8% (Table 3). For *A. alecto*, the divergence values for this species ranged from 0.0 – 3.9% distance. The genetic distance between the two species was between 5.4 – 12.1%. Overall, the mean divergence for the whole populations in Sabah and Sarawak was small, i.e. 0.3 – 0.7% as compared to 1.6% for the population in Kalimantan.

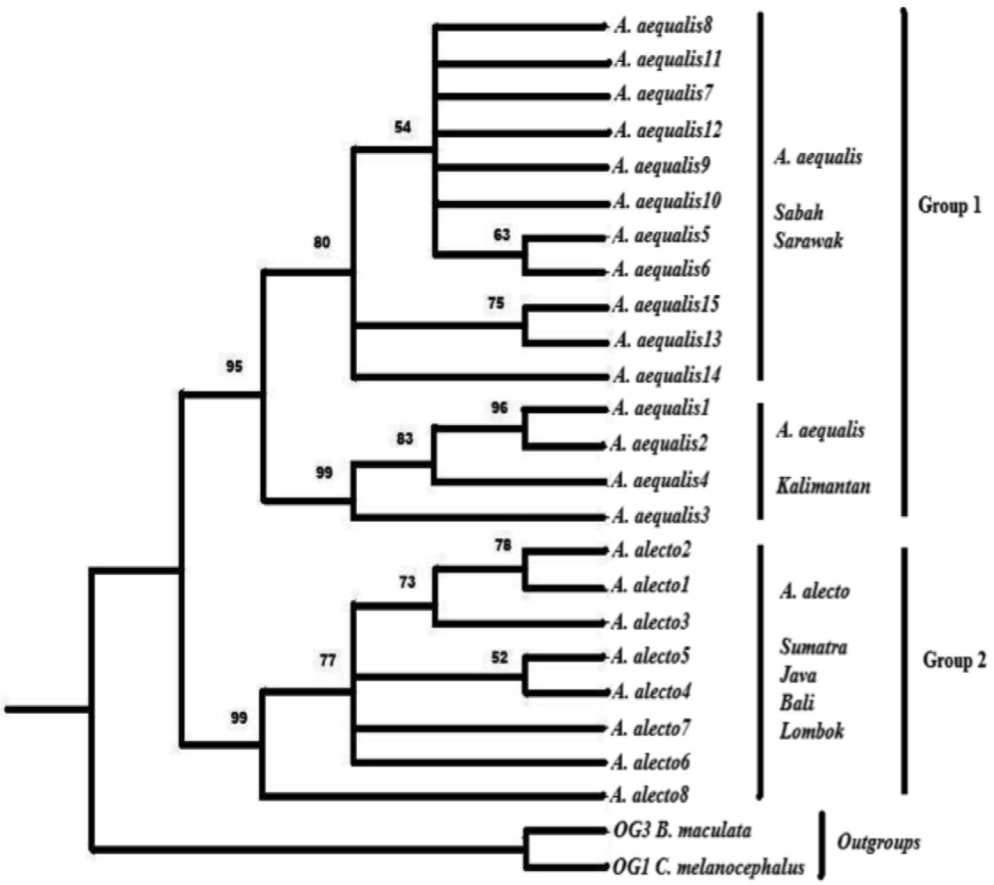

Fig. 2: Neighbour joining (NJ) the tree of genus *Aethalops* based on 290 bp 12S rRNA gene. Values on the branches were the NJ bootstrap estimates based on 1000 replicates and only >50% of the values are shown

TABLE 5
A summary of the variable sites, haplotype diversity, nucleotide diversity and the distribution of 15 haplotypes of 12SrRNA among the Bornean *A. aequalis* populations

Hap	Variable site	Populations				Whole populations
		Sabah	NSwak	SSwak	Kalimantan	
	1 1111111112 2222222223 3333333334 44] 1234567890 1234567890 1234567890 1234567890 12]					
Aae13	CCCTTGGGGC CCTCAAGTAC TAACTCTCGA GAGCGAACAC GC	(1)0.0556	(6)0.176			
Aae14	.... .T........ ....C. ..	(1)0.0556				
Aae5	....G....T C.G.......			0.444		
Aae6	....G..... C.G.......		(1)0.0294	(2)0.222		
Aae7	....G..... ..G.C.....		(1)0.0294			
Aae8	....G..... ..G.......	(17)0.833	(17)0.647	(3)0.333		
Aae12	.... T.C.GGAC.. ..G....... ....T.. ..	(1)0.0556				
Aae9	....G..... ..G...G...		(1)0.0294			
Aae11	TTTAAAACTA ....G..... ..G.......		(1)0.0294			
Aae10	....TG...G. ..G.......		(1)0.0294			
Aae15	....G.......		(1)0.0294			
Aae1	....G..... .CCT.T.GAG AG.AC.G..G ..				(1)0.2	
Aae2	....G..... .CCT.T.GAG AG.AC....G AG				(1)0.2	
Aae3	....G..... .CCT.T.GAG A..GA..... ..				(1)0.2	
Aae4	....G..... .CCT.T.GAG AGC.AG...G ..				(1)0.2	
K		1.307	1.519	1.056	4.600	3.225
Hd		0.31373±0.138	0.561±0.092	0.722±0.097	0.90000±0.161	0.615±0.067
Pi†		0.00451±0.00232	0.00524±0.00193	0.00364±0.00054	0.01586±0.00289	0.01096±0.00262

Note:
Dots indicate similar with Aea13 haplotype sequence
Numbers in the parenthesis indicate the number of individuals possessing that haplotype
NSwak – Northeast Sarawak; SSwak – Southwest Sarawak
N – number of sequence analysed; H – number of haplotypes; S – segregating sites; Sdiv – pairwise distance (estimated using Kimura-2-parameter) (Kimura, 1980); Hd – haplotype diversity; Pi – nucleotide diversity; K – average number of nucleotide differences.
† - sites with gaps were excluded

The alignment of the partial 12S rRNA gene successfully extracted eight haplotypes of *A. alecto* (Aa) and 15 haplotypes were *A. aequalis* (Aae) (Table 4). *A. alecto*1 to *A. alecto*8 were haplotypes of *A. alecto* from the populations in Lombok, Bali, Sumatra and Java. *A. aequalis*1 to *A. aequalis*4 were unique haplotypes of *A. aequalis* from the populations in Kalimantan. The remaining haplotypes of *A. aequalis* (*A. aequalis*5 – *A. aequalis*15) were the haplotypes from the mixed populations from Sabah and Sarawak. In particular, *A. aequalis* had three shared haplotypes and the most common haplotype was *A. aequalis*8 which was shared by the populations inhabiting Mt Kinabalu, Mt Trus Madi, Mt Murud, Mt Mulu, Mt Penrissen and Mt Pueh.

All the phylogenetic trees produced similar results by grouping the *Aethalops* into two different major clades (NJ, 83% MP, 79% ML, 89% BPP). Group 1 consists of *A. alecto* from Lombok, Java, Bali and Sumatra, while Group 2 consists of individuals from Borneo. Both NJ and MP separated Sabah and Sarawak into different groups from the population in Kalimantan, as supported by 95% and 92% of bootstrap value respectively in Group 2 (see Fig.2 and Fig.3).

Both ML and Bayesian trees (see Fig.4 and Fig.5) were constructed based on the

TABLE 6
The analysis of molecular variance (AMOVA) on the geographical population differentiation in Bornean *A. aequalis* using 12S rRNA gene

	Variance component	Variation (%)	Fixation Index, Φ	p[a]
Among groups	1.24494	53.36	Φ_{ct} = 0.53357	0.32454
Among populations within groups	0.28843	12.36	Φ_{sc} = 0.26503	0.00098*
Within populations	0.79987	34.28	Φ_{st} = 0.65718	0.00000*

* significant ($p < 0.05$)
[a] Probability of finding a more extreme variance component
Φ index than the observed by chance alone after 1000 permutations

TABLE 7
The Genetic differentiation matrix of the populations as measured by Φ_{st} and p-value (parenthesis) among the populations of *A. aequalis*

	Sabah	Northeast Sarawak	Southwest Sarawak	Kalimantan
Sabah	-			
Northeast Sarawak	-0.00734 (0.52252)	-		
Southwest Sarawak	0.32445 (0.00000)*	0.29194 (0.00000)*	-	
Kalimantan	0.84382 (0.00000)*	0.84956 (0.00000)*	0.83169 (0.00000)*	-

*significant ($p < 0.05$) with 1000 permutations

TABLE 8
Above diagonal are the measures of population subdivision (F_{st})* and gene flow (number of migrant, N*m*)* in parenthesis. Below diagonal are the measures of the nucleotide subdivision (N_{st})** among the populations of *A. aequalis*

	Sarawak		Sabah	Kalimantan
	Southwest Swak	Northeast Swak		
Southwest Swak		0.32092** (0.53)	0.33548 **(0.50)	0.79376* (0.06)
Northeast Swak	0.32101		-0.00550ns(-45.73)	0.76905 ***(0.08)
Sabah	0.34578	-0.00528		0.77756 **(0.07)
Kalimantan	0.79829	0.77357	0.77987	

Probability test (Chi-squared): *p < 0.05, **p < 0.01, ***p < 0.001, ns – not significant based on 1000 permutations of the sequence datasets.
*F_{st} and N*m* following Lynch and Crease (1990).
**N_{st} following Hudson *et al*. (1992).
Swak – Sarawak.

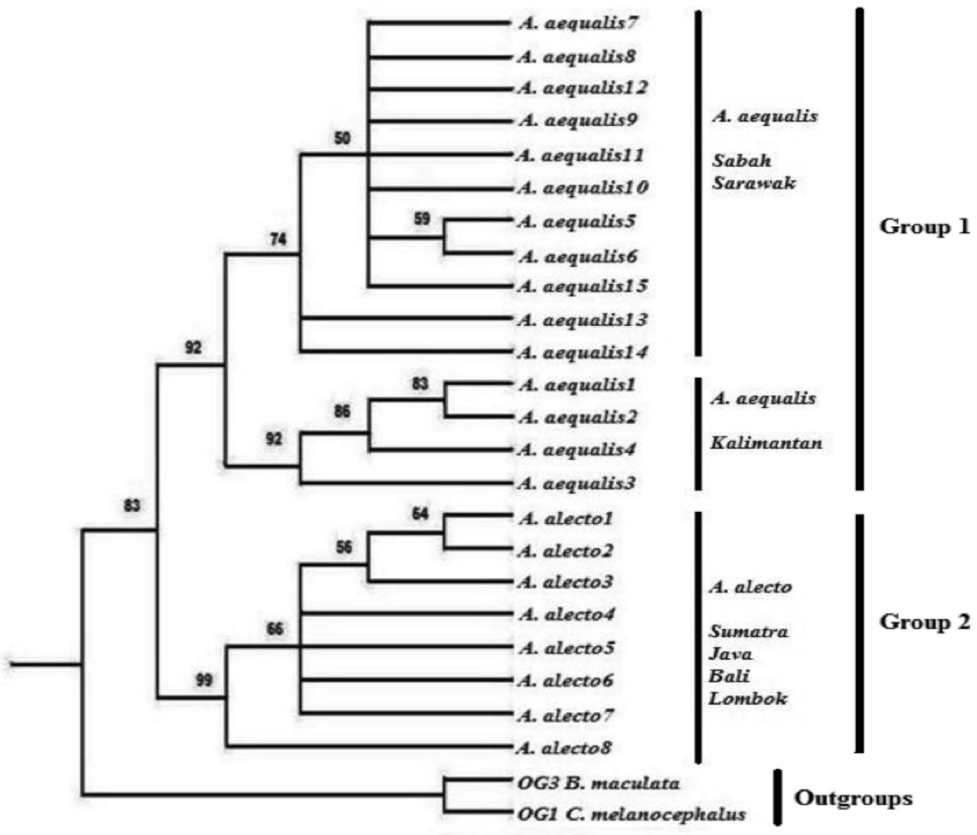

Fig.3: An equally weighted and rooted maximum parsimony (MP) tree of the genus *Aethalops,* based on 290 bp of 12S rRNA gene (tree length = 103; Consistency index, CI = 0.8447; retention index, RI = 0.9121). Values shown on the branches were the MP bootstrap estimates, based on 1000 replicates (only >50% of the values are shown)

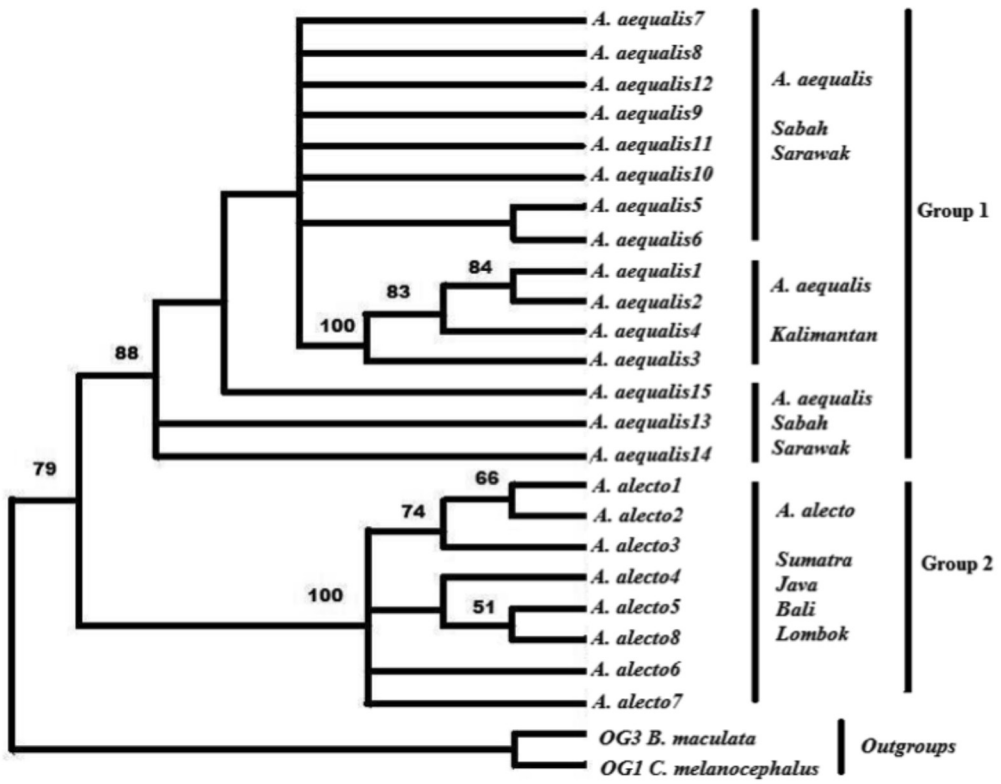

Fig.4: The maximum likelihood (ML) tree of 290bp 12S rRNA of genus *Aethalops* in Borneo with -Ln likelihood = 982.9361. Values shown on the branches represent the ML bootstrap value estimates, with 100 replicates (only >50% values are shown)

HKY+G substitution model, i.e. the best fit evolutionary model given by AIC in Modeltest 3.07 (Pasoda, 2005) with (-1nL = 982.9361; Ti/tr ratio = 2.0538; invariable sites = 0; among-site rate heterogeneity = 0.3642). Both ML and the Bayesian trees produced slightly different topologies from the NJ and MP trees. The Kalimantan group was clustered in between the Sabah and Sarawak clades. However, the grouping of Kalimantan in a group was strongly supported by high bootstrap value in all the phylogenetic trees (99% NJ, 92% MP, 100% ML, 1.00 BPP). As a conclusion, individuals that were obtained from Sabah and Sarawak were found to clade together with *A. aequalis* of Kalimantan. Therefore, it can be concluded to confirm that *Aethalops* from Sabah and Sarawak are *A. aequalis*.

The phylogenetic structure among the *Aethalops* was revealed by clustering in a minimum spanning network (MSN) (Fig.6). Based on this unrooted network of 12S rRNA gene, the *A. aequalis* from Borneo were successfully separated into two groups. Most of the haplotypes were

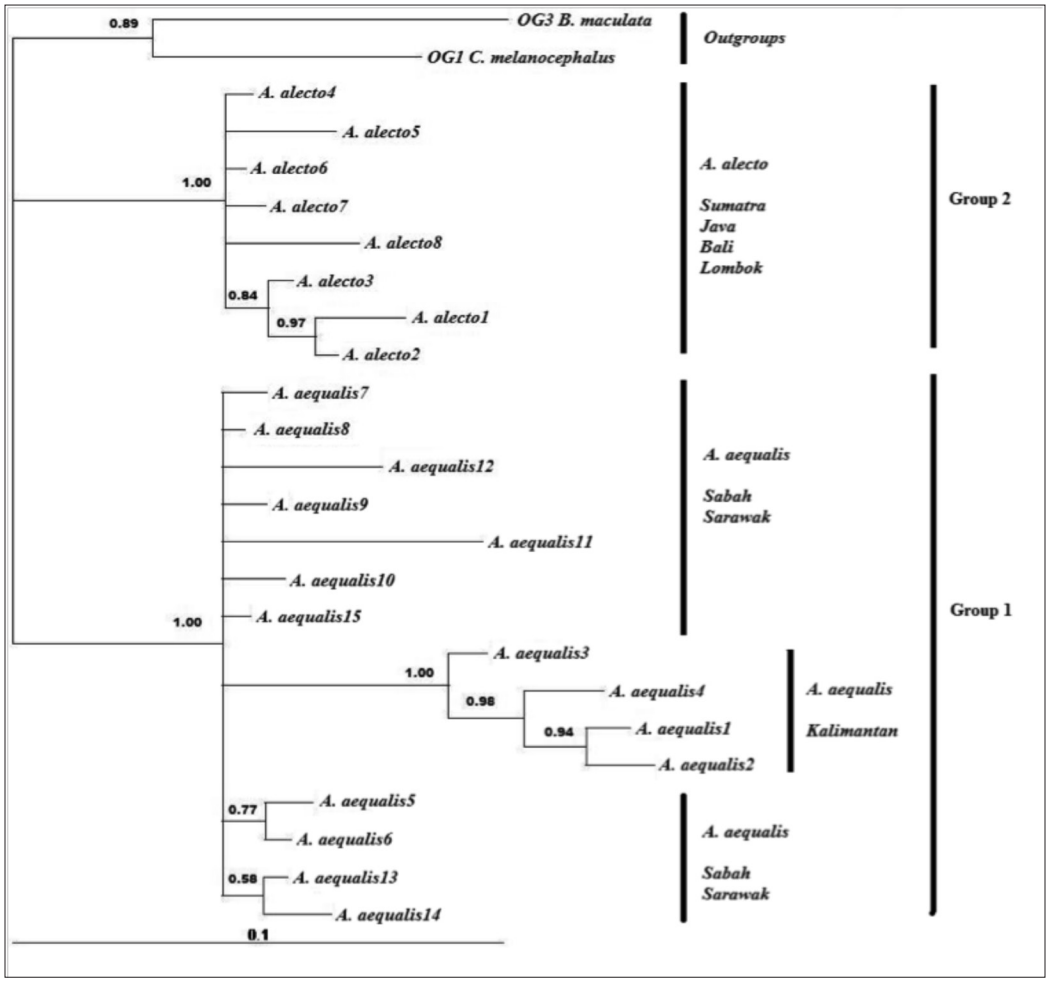

Fig.5: The Bayesian inference with 50% majority rule consensus tree of 12S rRNA gene of genus *Aethalops* in Borneo. Values of the Bayesian posterior probabilities (BPP) are shown on the branch nodes

unique haplotype for single population. *A. aequalis*8, *A. aequalis*6 and *A. aequalis*13 were shared by a few individuals from different populations. The frequencies of haplotype for each species were denoted by the proportional size of their haplo-nodes. In particular, *A. aequalis*11 from Bario was deviated from *A. aequalis*8 by 14 mutational steps. Nonetheless, the population in Kalimantan does not have sharing haplotypes with other populations of *A. aequalis,* either from Sabah or Sarawak. The genetic linkage between the two groups was deviated from *A. aequalis*3 of the Kalimantan population by 10 mutational steps before reaching the *A. aequalis* populations of Sabah and Sarawak.

The Genetic Analysis within the Population of the Bornean Pigmy Fruit Bats

Nucleotide divergence from 12S rRNA gene intrapopulation was also low

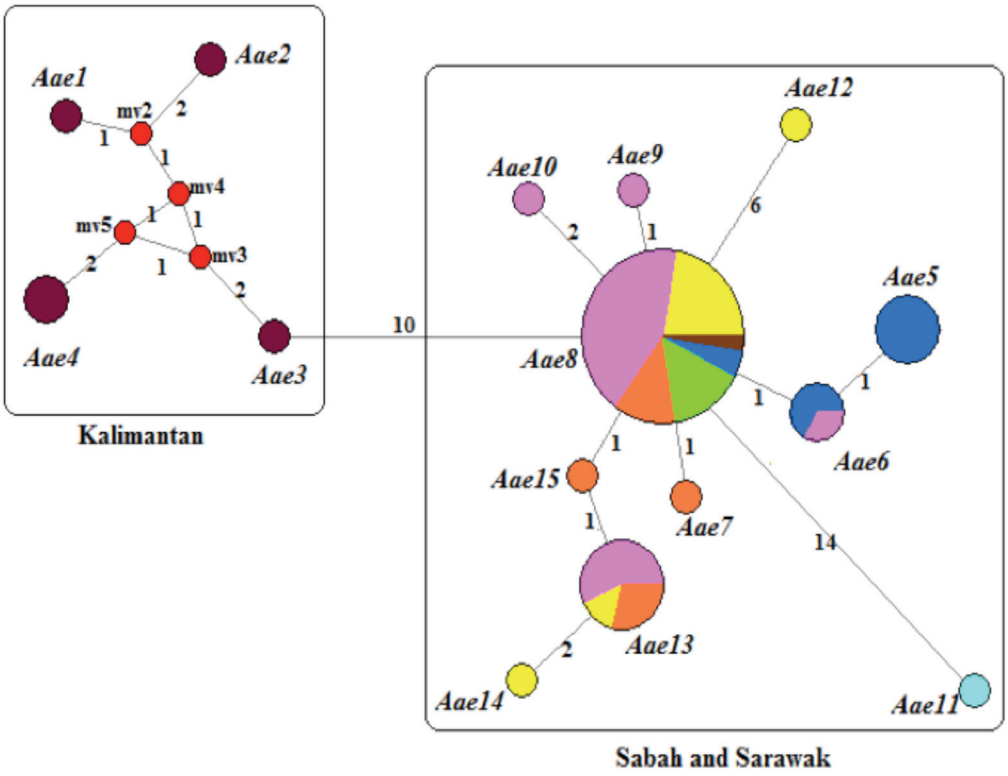

Fig.6: The haplotype mapping of 15 assigned haplo-nodes within eight populations of *A. aequalis* from Borneo. Each coloured nodes represents each population. Yellow node-Mt Kinabalu, green-Mt Trus Madi, pink-Mt Murud, orange-Mt Mulu, turquoise-Bario, blue-Mt Penrissen, brown-Mt Pueh, and purple-Kalimantan. The red nodes represent missing or unsampled haplotypes in this analysis. Note that each node represents a unique haplotype and the node sizes are proportional to the haplotype frequencies of the given population. Bold numbers indicated at the node branches are the number of mutational steps to connect the nodes. The minimum-spanning network (MSN) was generated by Network 4.5.1.6 programme (Fluxus Tech., 2004-2009)

as it ranged from 0.4 – 0.5% and net nucleotide divergence (0.003 – 0.2%) from the populations of Sabah and Sarawak, suggesting that these two populations had very high genetic similarities. The haplotype diversity of 12S rRNA was high, i.e. varying from 31.3% (Sabah) to 90.0% (Kalimantan). Nucleotide diversity of 12S rRNA gene interpopulation was low, ranging from 0.3 – 1.5%. Overall, 12S rRNA gene had a low level of genetic differences which may due to high frequency of haplotype *A. aequalis8* in the populations of both Sabah and Sarawak (33.3 – 83.3%) but not the populations of Kalimantan (Table 5). Another possible reason was that the samples used in this present analysis were small in number. According to Esa *et al*. (2008), the small sample size may underestimate the actual haplotype distribution among the study species.

A lack of significant relationship was observed between the geographical distance and net percent nucleotide divergence, Da (r = 0.023237; p = 0.622), among the populations of *A. aequalis* in Borneo (Fig.7). Hence, it indicated that the distance between the populations was not a factor that contributed to the divergence of the sequences in *A. aequalis*.

Population Subdivision

In the AMOVA analysis, the populations were grouped into three which consisted of Sabah population (Group 1), Northeast and Southwest Sarawak (Group 2) and Kalimantan (Group 3). The results showed that the among group has the highest variation with 53.36% and was not siginificantly differentiated (p = 0.32454), followed by within the populations with 34.28%, which were to be highly significant (p = 0.0000), and lastly among the populations with 12.36% (p = 0.00098) of the variation differentiated the individuals (Table 6). The estimated Φ_{st} values among the grouped populations were significant for the genetic differentiation matrix of the populations (Table 7).

The levels of nucleotide (N_{st}), population subdivision (F_{st}) and migrants per generation (Nm) are presented in Table 8. The results show that the Bornean populations of *A. aequalis,* except for those in Kalimantan, have low levels of N_{st} and F_{st}, with high

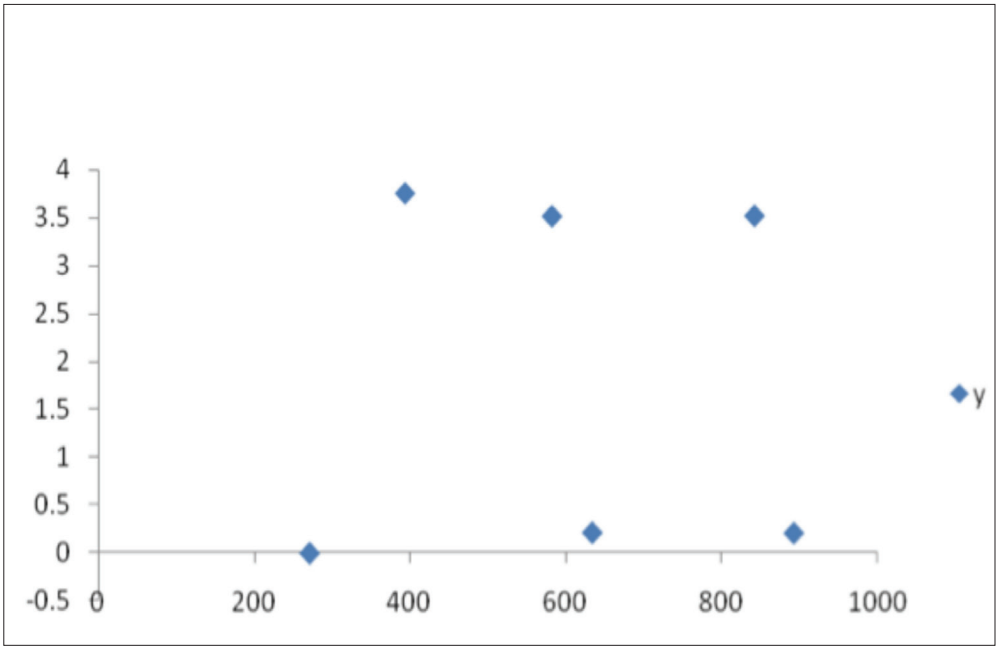

Fig.7: Scatter plots showing the relationships of the geographical distance and net nucleotide divergence, and Da (%) between the populations of *A. aequalis* in Borneo. Regression statistic: y = 0.00054; correlation coefficient, r = 0.023237)

levels of N*m*. Meanwhile, the lowest level of N_{st} and F_{st}, and the highest level of N*m* are shown by the Sabah-Northwest Sarawak population, indicationg that the gene flow between these populations is the highest. The Kalimantan population appeared to have the lowest gene flow, suggesting that this population was isolated from Sabah and Sarawak, despite being on the same island (N*m* = 0.06 – 0.08). The comparisons of genetic differentiation among the populations of Bornean *A. aequalis* were significant ($p < 0.01$ and and $p < 0.001$), except for the genetic differentiation between Northeast Sarawak and Sabah. A highly significant genetic differentiation ($p < 0.001$) was also observed between Kalimantan – Northeast Sarawak.

DISCUSSION

Overall, the phylogenetic trees NJ, MP, ML and Bayesian successfully resolved genus *Aethalops* into two major monophyletic groups corresponding to *A. alecto* and *A. aequalis,* with high bootstrap values (69% NJ, 83% MP, 79% ML, 1.00 Bayesian). The interpopulation relationships of *A. aequalis* from Sabah and Sarawak were mixed up; however, the separation between Malaysian Borneo (Sabah and Sarawak) from those in Kalimantan was clearly distinct, except for ML and Bayesian in which Kalimantan was clustered in between two clades consisting of Sabah and Sarawak. Thus, it is conclusive that the species in Sabah and Sarawak were confirmed to be *A. aequalis*, and should no longer be referred to as *A. alecto*, and *A. aequalis* is classified as a distinct species from *A. alecto*. The findings of the present study support those of Kitchener *et al*. (1993) and Maharadatunkamsi *et al*. (2006). The genetic distance within *A. aequalis* among the Malaysia Borneo is small, with a mean divergence (0.3 – 0.7%) that is almost consistent with that of Faisal (2008) with 0.2% using Cyt. *b*.

The interpopulations mixing of Sabah and Sarawak indicated that the populations of *A. aequalis* have had high genetic similarities. Among the *A. aequalis* haplotypes from Sabah and Sarawak, *A. aequalis8* or Aae8 is the most common shared haplotypes among the individuals, ranging from southwest Sarawak to northeast Sarawak and Sabah. Apart from being genetically similar, they are considered as a single morphotype based on the same samples taken from the populations in Sabah and Sarawak, as indicated by another study using skull morphometric analysis. The slight morphological difference between southwest Sarawak and northern Borneo populations of these bats is possibly due to the adaptation to food resources to survive in the species competition. In more specific, the skull of the bats may have evolved to adapt into optimised form to meet the demand of holding and masticating of different food sources, depending on what habitat provides (Tingga, 2010).

Based on coalescent theory, the most common haplotype may be the oldest, with the expectation that the haplotype should be geographically widespread. Therefore, *A. aequalis8* is predicted as the ancestral haplotype. However, since *A. aequalis8*

is not identified as basal by the outgroup rooting, it is suggested that this may not be the absolute oldest haplotype, but it can relatively be considered as one of the ancestral haplotype as compared to other observed haplotypes of *A. aequalis*. A relatively similar case was also observed within the song sparrow haplotypes (Fry & Zink, 1998), where two common haplotypes were observed to be widely distributed but not placed at the basal clade to be considered as the oldest haplotype. The present study also showed that the common haplotypes were not rooted at the basal; however, *A. aequalis13* and *A. aequalis14* were rooted at the basal of the monophyletic group of *A. aequalis* from Sabah and Sarawak.

*A. aequalis*11 (Bar3) was genetically distance from other haplotypes (genetic distance 3.9 – 5.8%). Bario (Kelabit Highland) is situated at the geographical boundary between Kalimantan and Sarawak. This individual is genetically unique and has high genetic distance ranging from 1.1 – 4.6%). Moreover, this individual may still retain its ancestral haplotype which diverged million of years ago (mya). Such a divergent individual from this species is regarded to be associated with distinct geographic ranges which reflect a long-term zoogeographic barrier to gene flow that is largely independent of glaciations events (reviewed by Avise *et al*., 1987, as cited in Dobson *et al*., 1995).

Using the constant transversion rate of 0.27% per million years (Mindell *et al.,* 1991), the separation time between *A. aequalis* and *A. alecto* was estimated approximately 12 mya, which fell during the mid Miocene period. The data obtained for the speciation of *A. aequalis* were not consistent with the Pleistocene speciation hypothesis. Therefore, distributional of this species was apparently due to dispersal rather than vicariance, changes of sea level or vegetational change. According to Haq *et al*. (1993), however, there was a relatively low sea level even before the last 2 mya.

Thus, the question now is that which of the observed islands in Indonesia was the earliest population of *A. alecto* after its separation from *A. aequalis*? As discussed earlier, the divergence time between *A. alecto* and *A. aequalis* was ~12 mya, which predated the Pleistocene period in Lombok (the earliest ~13.2 mya), followed by Java and Bali (~12.53 mya) and Sumatra (~10.96 mya). Hence, Lombok was predicted to be the ancestor population among all the *A. alecto* populations that had been observed. Hypothetically, Lombok was the first colonised island of *A. alecto* after this particular species had diverged from *A. aequalis*. Nonetheless, it is still undetermined whether the two forms of *Aethalops* arose from Borneo or Lombok before it diverged.

It is possible that *A. alecto* from Sumatra, Java, Bali and Lombok, following 12S rRNA constant transversion rate (Mindell *et al*., 1991) started to disperse from Lombok approximately ~1.9 mya, based on the 12S rRNA gene mammals divergence time by Mindell *et al*. (1991) to Sumatra, and it then spread to Java, Bali and Lombok (Lombok-Java = ~0.89 mya, Lombok-Bali =

~0.89 mya, Lombok-Sumatra = ~1.98 mya, Sumatra-Java = ~0.74 mya). The prediction time for *A. aequalis* in Java is supported by the findings of van der Bergh *et al*. (2001), who found no evidence indicating mammals present on Java prior to 2.4 mya. After that time, intermittent land bridges allowed colonisation to occur (van der Bergh *et al*., 2001). It was during the early Pleistocene that the presence of the fauna characteristic of open woodlands found in the vertebrate fossil record of Java (van der Bergh *et al*., 2001). At this stage, there was a connecting tract of open vegetation from the Asian mainland to Java. According to Bird *et al*. (2005), the earlier separation would have likely caused the island to retain a group representative from the populations frequenting the area during the glacial times.

In the analysis of the current data, all the phylogenetic trees showed a close genetic relationship between the populations in Lombok and Bali. A drop of sea level in the Strait of Lombok would have likely facilitated the dispersal of *A. alecto* to Bali across the Lombok Strait. Similarly, this condition has also been observed in other species of bats, such as *Myotis muricola* and *Cynopterus brachyotis*. A recent study by Wiantoro (2010) indicated that in Bali and Krakatua, *M. muricola* was not clustered accordingly to the population groups based on Wallace's Line. Similarly, Wiantoro (2010) also stated that the low sea level in the Strait of Lombok had provided the possibility of gene flow within *M. muricola* Eastern and other populations on Krakatau and Bali islands to the populations on other Lesser Sunda islands.

Historical Population of the Bornean Pigmy Fruit Bats

Using the constant transversion rate of 0.27% per million years (Mindell *et al.,* 1991), *A. aequalis* was found to have diverged from its sister species *A. alecto* approximately 12 mya during the mid-Miocene period. Therefore, it was hypothesised that the widespread distribution of *A. aequalis* in Borneo was most probably due to the colonisation event that occurred before the Pleistocene and was not caused by the changes in the sea level (Dobson *et al*., 1995). It was also suggested that the species is endemic to the island and this originated > 2 mya. However, the species has not undergone repeated extinction and recolonisation, and it is more likely to have persevered at a particular island since its origin (Steppan *et al*., 2003). Thus, Kalimantan (West Central Kalimantan at Taman Nasional Bukit Baka) has been predicted to be the location of the original population of *A. aequalis*. Furthermore, among the populations of *A. aequalis* in Borneo, the population in Kalimantan was found to be the ancestor towards the other populations observed in Borneo.

There was a very high genetic difference between the populations in Sabah and Sarawak compared to the one in Kalimantan, with a high genetic distance of 3.5 – 8.8% and very low level of gene flow. The Tamo Abo Range is the boundary that separates Sarawak and Sabah from Kalimantan and thus limits the gene flow between the population of Malaysian Borneo and Kalimantan. Hypothetically, the specimens from Sabah and Sarawak could also be a

sub-species of the population in Kalimantan. Nevertheless, this has yet to be investigated as there were no secondary data available in the present study to support this hypothesis. According to Fry and Zink (1998), DNA polymorphism has been used as an inference on the historical patterns of population expansion.

Based on the flow of the divergence time from 12S rRNA gene, it was hypothesised that the pattern of movements of this particular species went from Kalimantan to southwest Sarawak to the northeast of Sarawak and Sabah. Since the divergence from *A. alecto* (12 mya), the population of *A. aequalis* from Kalimantan dispersed to southeast Sarawak after approximately 5 million years (7.2 mya) and later from southwest to northeast of Sarawak (370 ka) and Sabah (370 ka). Both the populations of the northeast Sarawak and Sabah were recently diverged from the one in southeast Sarawak (see Fig.8). Meanwhile, age estimation between northeast Sarawak and Sabah groups suggests that the northern populations of Borneo are sister populations that are supported by very close genetic relationship and high gene flow.

The divergence time between southwest Sarawak and northeast Sarawak –Sabah groups occurred during the Pleistocene. This suggests that the haplotypes from Mt Mulu, Mt Murud, Mt Kinabalu and Mt Trus Madi have recently diverged from one another. In fact, this could be the reasons why these populations are highly genetically similar. The population in southwest Sarawak was predicted to be the ancestral group because during the Pleistocene period, The Northern Borneo was suggested to act as a refugium for the lowland rainforest species during the late Pleistocene (Brandon-Jones, 1998; Garthorne-Hardy *et al*., 2002). The Pleistocene facilitated the dispersal and genetic exchange of *A. aequalis* populations that are now confined to mountaintops.

In general, the mountains in Sabah and Sarawak form the backbone of a highland ranging from Mount Kinabalu (Crocker range) through Kelabit Highland (Bario) to Madi Plateau and the Schwaner Range of Kalimantan. It is assumed that the dispersal of a montane bat is similar to a montane bird, where dispersal to another mountain or range occurs along a spinal chain that connects the mountains. Most mountains in Sabah and Sarawak are connected along a chain, with an elevation of more than 1500 m a.s.l. Along this chain, the ridge breaks away to form a long stretch of lowland (Lubok Antu) between south western Sarawak and Mount Lawit. The separation of this ridge could be one of the reasons that had led to genetic divergence between the population at Mt Penrissen and those in the northern part of Sarawak and Sabah.

CONCLUSIONS

In conclusion, 12S rRNA was found to be able to resolve the interspecific relationships of *A. aequalis* and *A. alecto*. The current findings conclude that *A. aequalis* is a single unit panmictic population in Borneo and thus support the previous studies that *A. aequalis* is no longer known as a subspecies of *A. alecto*. Moreover, the

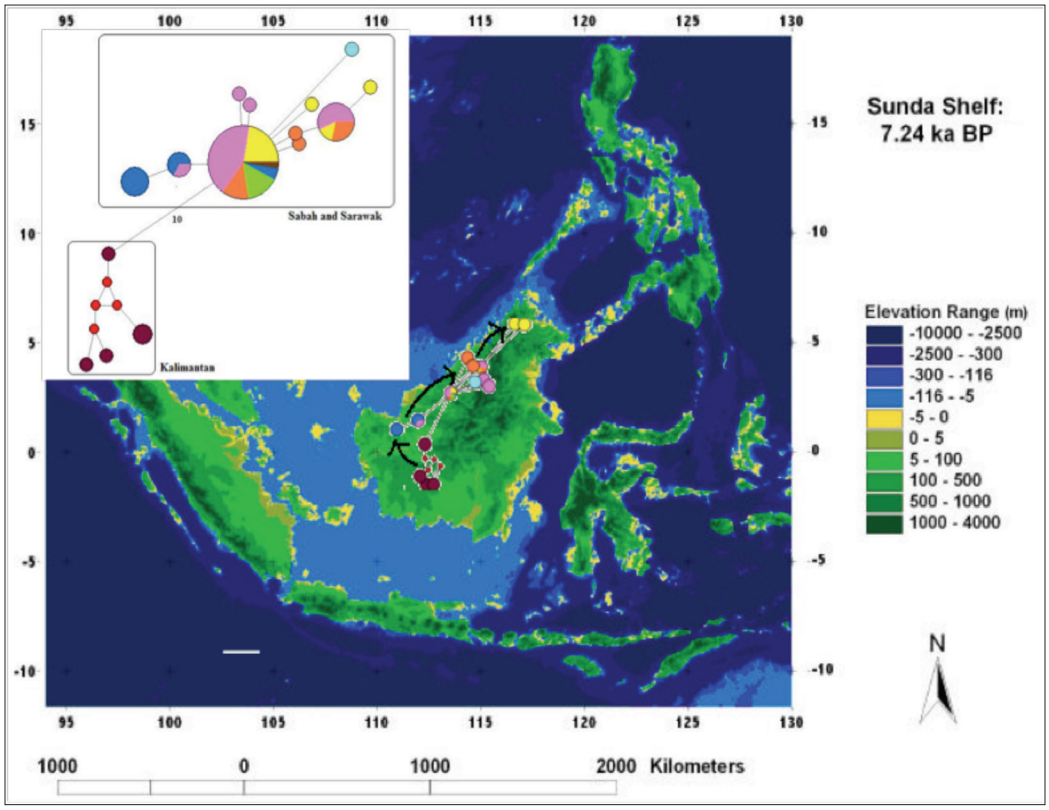

Fig.8: The Minimum Spanning tree of 12S rRNA haplotypes (left) and the hypothetical origin population of *A. aequalis* and its route dispersal, as indicated by the arrows (right). The map was adapted from Sathiamurthy and Voris (2006)

genetic distance between *A. aequalis* from Malaysian Borneo and Kalimantan is rather high and this is supported by the high value of population and nucleotide subdivision, which produced a new hypothesis on this particular species, with the possibility of two subspecies within Borneo. However, this gene is unable to resolve the intraspecific relationships of *A. aequalis* in Sabah and Sarawak. The intermixing population among the populations of *A. aequalis* in Sabah and Sarawak indicates high genetic similarities, whereby the dispersal was hypothesised from southern Sarawak to the northern Borneo, and that this dispersal was facilitated by Pleistocene climatic fluxes. The population in Kalimantan was also postulated as the possible ancestral for *A. aequalis* of Borneo.

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Hibiscus sabdariffa Aqueous Extracts Prevents Progression of Acute Liver Injury Induced by Acetaminophen

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ABSTRACT

Hibiscus sabdariffa (local name Roselle) is usually used as a beverage in Southeast Asia. It has been shown that this plant has benefits to the health in term of improving diabetes and hiperlipidemia conditions. In this study, the effect of *H. sabdariffa* aqueous extracts in preventing acute liver injury progression in rats induced by acetaminophen (or paracetamol, PCM) was investigated. Results of the current study showed that intravenous injection of PCM at 1000 mg/kg induced lipid proxidation (malonaldehyde, MDA) and deteriorated liver marker enzymes (alanin transaminase, ALT and glutathione S-transferase, GST), as well as liver glutathione (GSH) and liver morphology. Feeding *H. sabdariffa* extract orally (500 or 1000 mg/kg) for three days after the PCM treatment was found to have significantly reduced lipid peroxidation. The depleted GSH observed in the affected liver returned to almost normal, while the liver marker enzyme, ALT and GST levels were improved by giving the extract. In histological examination, the *H. sabdariffa* extract was shown to have reduced the incidence of liver damage. However, a high dose of *H. sabdariffa* treatment to the untreated rats increased liver MDA and GST and serum ALT levels, although at a much lower level than the PCM-treated rats. Hence, the liver histology of these rats remains normal. In conclusion, the current study has shown that the post-treatment of *H. sabdariffa* prevents the progression of acute liver damage induced by PCM. However, the consumption of the plant at high dosage should be taken with caution.

Keywords: Hibiscus sabdariffa, paracetamol, liver toxicity, MDA, GSH, GST, ALT

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INTRODUCTION

Hibiscus sabdariffa or roselle is also known as *asam paya, asam keling, asam susur* or

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asam belanda, and it can be found in Asian and African tropical forests. Lately, it was planted in big scale in Malaysia for its calyx to make juice, syrup, jam, marmalade and chutney. Calyx is part of the plant's flower that enlarges and turns into fleshy and juicy structures once the flower wilts. Nutritional values of the calyx have long been established (Mat Isa, 1985). It is rich in vitamin C and also contains vitamins D, B1, B2 and B complex. Interestingly in some countries, their uses are not restricted to food industries only, as they are also used in traditional medicine to treat certain diseases. In India, Africa and Mexico, all the above-ground parts of the roselle plant are used in native medicine. Infusions of the leaves or calyces are regarded as diuretic, cholerectic, febrifugal, hypotensive, and can decrease the viscosity of blood, reduce fats in blood and stimulate intestinal peristalsis (Morton, 1987).

Some of these claims are proven to be true through scientific findings. Some recent studies have demonstrated that *H. sabdariffa* significantly reduced blood pressure in experimental animals (Ajay *et al.*, 2007) and humans (Herrera-Arellano *et al.*, 2004). In 1991, el-Saadany and colleagues confirmed the hypocholesterolemic activity of the plant when they observed lowering effect in the different lipid fraction levels of hypercholesterolemic rat. The continuous interests in this plant up to now have led to many scientific discoveries that exceed the traditional medicinal belief. One of them is the ability of the plant extract to protect liver from acute injury. In various studies, pre-treatment of *H. sabdariffa* extract to animals suffering from liver injury induced by tert-butylhydroperoxide (t-BHP), lipopolysaccharide and azathioprine (Liu *et al.*, 2002; Lin *et al.*, 2003; Amin & Hamza, 2005) was found to have blocked the elevated levels of liver marker enzymes (alanine aminotransferase, ALT and aspartate aminotranferase, AST) and improved the abnormality of liver histology. In addition, the extract also has the capability to protect liver from radiation (Adaramoye *et al.*, 2008) and counteract the over-dosage effect of acetaminophen (paracetamol, PCM) (Olaleye *et al*., 2010; Rocha, 2008). All these studies, however, showed that the liver damage could be reduced if one consumed *H. sabdariffa* before the occurrence of the damage. Conversely in this study, the researchers purposely damaged the liver through a treatment of excess PCM before supplementing the animals with *H. sabdariffa* aqueous extract. This was done to investigate whether *H. sabdariffa* extract would be able to prevent acute liver injury progression induced by the damaging agent. The effects of *H. sabdariffa* to a normal liver were also determined. To achieve these, the levels of liver marker enzymes, alanin transaminase (ALT) and glutathione S-transferase (GST), plus malonaldehyde (MDA) and glutathione (GSH), were investigated. In addition, the morphological changes of liver histology were also investigated.

MATERIALS AND METHODS

Animals and Diets

Thirty-six male *Sprague dawley* rats, weighing 190-200 g, were used in the experiment. They were given commercial rat pellets (Gold Coin Company, Malaysia) and water on a daily basis. The animals were equally divided into six groups, i.e. the untreated (control), PCM-treated (hepotoxicity animals), low *H. sabdariffa* plus PCM diet-treated, high *H. sabdariffa* plus PCM diet-treated, as well as low and high *H. sabdariffa* diet-treated groups.

The control group was given the commercial rat chow diet throughout the experiment. Liver toxicity was induced in the animals by giving 1000 mg/kg body weight animal PCM intraperitoneally. The mixed diet- and low and high *H. sabdariffa* diet-treated groups were given 500 and 1000 mg/kg body weight animal *H. sabdariffa* extract, respectively. It was given orally once for three consecutive days. For the mixed diet-treated animals, the extract was given after the PCM injection. The extract was prepared according to the method of Azuine *et al.* (1992). Dried *H. sabdariffa* calyx used in the preparation of the extract was supplied by Monrose Company Sendirian Berhad, Kuala Lumpur.

Collections of Serum and Liver

After 72 hours of the treatment, the animals were fasted overnight in preparation for the serum and liver collections. In the morning, the animals were weighed and anesthetized under chloroform, while the thoracic abdominal cavity was opened. Blood was collected by heart puncture and serum was separated by centrifugation of the blood. The liver was excised from each animal and washed with 1.15% cold calcium chloride to remove the blood stain. The liver was weighed and a small section of the liver was fixed in 10% (v/v) formalin solution for hematoxylin and eosin (H & E) staining. 750 mg liver was weighed for GSH assay and the rest was homogenized for the total protein, MDA and GST level determination.

Biochemical Analyses

The total protein for both the serum and liver was determined using Bradford's method (1976). Serum ALT and liver GST levels were estimated according to Reitman and Frankel (1957), and Habig *et al.* (1974), respectively. Liver GSH and MDA levels were determined following the method of Hissin and Hilf (1976) and Ledwozyw *et al.* (1986), respectively.

Histology

The liver sections that were fixed in 10% formalin solution were processed for the normal histological section. The tissue samples were sectioned, stained with H & E and examined under light microscope for observation of morphological abnormality.

Statistical Analysis

The mean values obtained in the biochemical analyses were analyzed for the statistical difference using the Student's *t*-test.

TABLE 1
The effect of roselle or *H. sabdariffa* on the total protein level in the serum and liver.

Treatment group	Serum	Total protein (mg/ml) liver
Untreated (control)	3.01 ± 0.01	18.42 ± 0.28
Paracetamol (1000 mg/kg)	1.68 ± 0.01*	15.68 ± 0.34*
500 mg/kg roselle & paracetamol	3.35 ± 0.02**	15.58 ± 0.03
1000 mg/kg roselle & paracetamol	3.93 ± 0.10**	18.88 ± 0.60**
500 mg/kg roselle	3.62 ± 0.06*	18.43 ± 0.22
1000 mg/kg roselle	4.24 ± 0.19*	23.35 ± 0.10*

The data are presented as the mean ± SEM of 6 animals. *Significantly different compared to the untreated group (control), $p<0.05$. **Significantly different compared to the group given paracetamol, $p<0.05$.

RESULTS AND DISCUSSION

Total Protein Analyses

Table 1 shows that the treatment of 1000 mg/kg PCM to the rats significantly reduced 44% and 15% of the total protein in the serum and liver, respectively, as compared to the untreated rats. Similar observations were also reported by Lu (1985) who observed that the metabolism of excess PCM in the liver led to the formation of excess free radicals and reactive metabolite, N-acetyl-p-benzoquininimine (NAPQI) that formed covalent bond with the protein thiol group which eventually disrupted the synthesis of the protein and reduced the total protein level. When the right dose of the PCM was taken, NAPQI was detoxified by glutathione S-transferase into mercapturic acid, which was then excreted via urine to avoid it from affecting protein synthesis.

The results also showed that the total protein reductions in the PCM-induced hepatotoxicity rats seemed to be counteracted by giving high and low dosages of *H. sabdariffa* to the rats after the PCM treatment (Table 1). Similar observations were also made by Onyenekwe *et al.* (1999) who discovered that the addition of *H. sabdariffa* calyx infusion to the hypertensive rats had increased the total protein in the serum and liver. Interestingly, the addition of *H. sabdariffa* to the untreated rats also increased the total protein in both the serum and liver (Table 1). This increase is actually important in forming and repairing new and damaged cells and tissues, respectively.

Serum Alanin Transaminase Analyses

The increased level of serum ALT is normally used as an indicator of liver damage. During liver damage, cell lyses normally cause higher distribution of ALT in the liver cytoplasm which will eventually lead to the spill of ALT into the blood circulation (Galteau *et al.*, 1980). In this study, the PCM-treated rats showed 74.5% increase of serum ALT as compared to the control, indicating hepatotoxicity (Table 2). When *H. sabdariffa* was given to these rats after the PCM injection, a significant reduction of elevated ALT level was observed (Table 2). In more specific,

TABLE 2
The effects of roselle or *H. sabdariffa* on the ALT, GST, MDA and GSH levels.

Treatment Group	ALT (IU/l)	GST (IU/mg)	MDA (nmol/mg)	GSH (ug/g)
Untreated (control)	71.86 ± 5.22	0.70 ± 0.29	0.12 ± 0.02	0.28 ± 0.02
Paracetamol (1000 mg/kg)	125.37 ± 1.64*	12.38 ± 0.37*	8.66 ± 0.74*	0.056 ± 0.008*
500 mg/kg roselle & paracetamol	119.51 ± 1.85**	2.05 ± 0.33**	6.55 ± 0.34**	0.073 ± 0.005
1000 mg/kg roselle & paracetamol	81.55 ± 2.08**	1.15 ± 0.06**	4.96 ± 0.29**	0.21 ± 0.009**
500 mg/kg roselle	82.21 ± 2.58	2.74 ± 0.17*	0.35 ± 0.02*	0.26 ± 0.010
1000 mg/kg roselle	96.17 ± 3.41*	2.00 ± 0.17*	0.34 ± 0.02*	0.28 ± 0.009

The data are presented as the mean ± SEM of 6 animals. *Significantly different compared to the untreated group (control), $p<0.05$. **Significantly different compared to the group given paracetamol, $p<0.05$

at 1000 mg/kg, *H. sabdariffa* gave a larger reduction of ALT level as compared to the lower dosage of *H. sabdariffa*. In another study, Ali *et al*. (2003) found that feeding *H. sabdariffa* extract before the PCM treatment also resulted in a similar effect. Intriguingly, feeding high dose of *H. sabdariffa* to untreated rats significantly increased the ALT level although much lower compared to the PCM-treated rats (Table 2). This increase, however, does not change the morphology of the liver (Fig.1). Other study has also shown a similar observation when *H. sabdariffa* (at 250 mg/kg in 3, 5, 10 and 15 doses) was given to normal rats (Akindahunsi & Olaleve, 2003), in which the increase in ALT was not followed by pathological changes in the liver and the heart, when both the organ sections were observed under microscope. One report has suggested that only excessive doses of *H. sabdariffa* given for a relatively long period could have a deleterious effect to the rats, particularly on their testes (Ali *et al.*, 2005).

Liver Glutathione Analyses

Hepatotoxicity induces depletion of GSH (Ohta *et al.*, 1995), as higher amount of GSH is required to detoxify the toxic compounds. In this study, GSH level was significantly reduced by 80% in the PCM-treated rats compared to the control group (see Table 2). It was reduced due to the increased GSH requirement in conjugating with free radicals and NAPQI (PCM reactive metabolite) in order to detoxify it (Wendel *et al.*, 1979). The present study have also demonstrated that the post-treatment with *H. sabdariffa* at 500 mg/kg increased 31% of GSH level observed in the PCM induced-hepatoxicity rats and increased 275% (i.e. almost similar to the level of untreated rats) when given 1000 mg/kg of *H. sabdariffa* (Table 2). A similar observation was also demonstrated in another study when the pre-treatment of *H. sabdariffa* extract was given to a PCM-treated mouse (Liu *et al.*, 2010). It is important to note that the treatments of both low and high doses of *H. sabdariffa* to

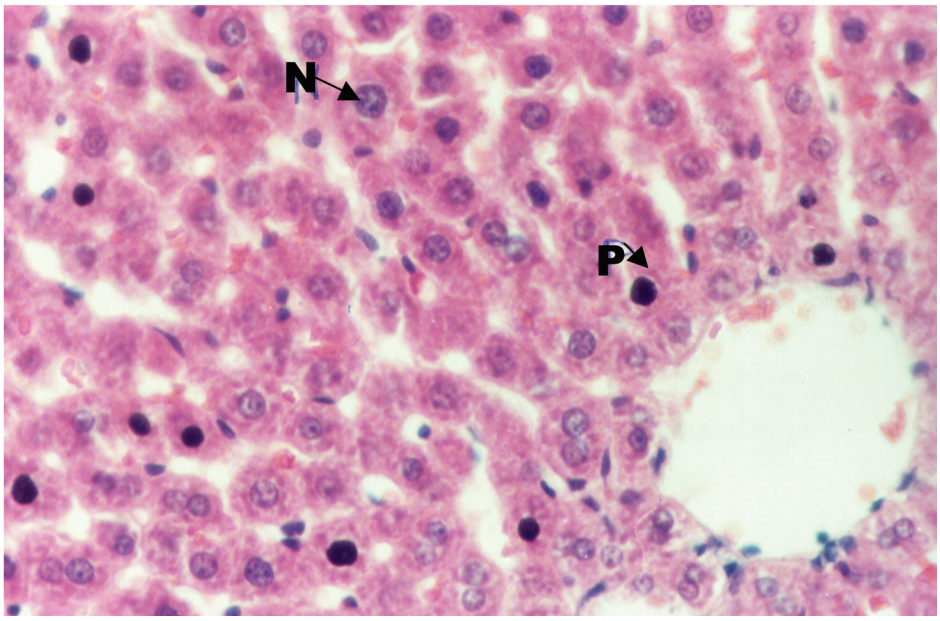

Fig.1: Liver of the PCM-induced animals (400x). The normal and picnotic nucleus are indicated by the arrow. N = nucleus; P = picnotic nucleus

the untreated rats did not increase the GSH level (Table 2). In fact, it was found to have been maintained at almost similar level of the normal rats. This finding suggests that there are no bioactive compounds in *H. sabdariffa* that stimulate the synthesis of GSH; instead, it is possible that the *H. sabdariffa* aqueous extract itself contains bioactive compound which removes PCM toxic metabolites, and thus increases the reduced GSH level of affected animals. In other studies, the bioactive compounds that have been confirmed to scavenge free radicals in *H. sabdariffa* include protocatechuic acid and anthocyanin (Tseng *et al.*, 1996; Wang *et al.*, 2000).

Liver Glutathione S-transferase Analyses

In this study, the liver GST activity was tremendously increased in the group treated with PCM (Table 2). This elevated level was anticipated as it was one of the mechanisms to detoxify the high dosage of PCM given. Chasseaud (1976) showed that GST catalysed the conjugation of GSH with the electrophilic groups of other toxic compounds. This reaction neutralized the toxic compounds and induced them to easily dissolve in water so as to be easily secreted into urine or faeces. Meanwhile, supplements of *H. sabdariffa* at low and high doses after the PCM treatment were found to have significantly reduced the elevated GST level (Table 2). This result confirms the above statement (in *Liver Glutathione Analyses*) that the bioactive compounds in *H. sabdariffa* help to remove undesired PCM toxic metabolites, and thus, reduce the burden of GST to detoxify them and the elevated GST level in the

PCM-treated animals. However, feeding *H. sabdariffa* at both doses to the untreated rats also increased the GST level, and this is similar to the reaction of the PCM-treated rats. Nonetheless, the increase was not alarming and it was most probably because a few chemical constituents in *H. sabdariffa* at 500 and 1000 mg/kg animal were too high and needed to be removed from the liver and thus caused the increase in the GST level.

Liver Malonaldehyde Analyses

MDA is one of the products of lipid peroxidation. By determining the MDA level, the stage of lipid peroxidation could be estimated. Normally, an elevated level of MDA indicated a high lipid peroxidation activity and reduced the level of GSH (Albano *et al*., 1983). The results showed that the treatment of PCM to the rats drastically increased the MDA level (Table 2), confirming the high lipid peroxidation activity in the affected liver. Interestingly, this extreme elevated level was counteracted by giving *H. sabdariffa* in both doses. The supplement of both high and low doses of *H. sabdariffa* to the untreated rats significantly increased the MDA level as compared to the control group, although this was at much lower level than the PCM-treated rats. It is intriguing to discover that although *H. sabdariffa* can reduce the lipid proxidation caused by over-dosage PCM, it can also cause lipid peroxidation in the untreated rats. Even though the exact reasons for these two effects are not known, these are probably due to the various effects of the many chemical constituents that are present in the crude extract of the plant.

Based on the results of both GST and MDA, future studies must be carried out to determine the right dosage of *H. sabdariffa* consumption and the length in which the supplement should be taken so as not to deteriorate the general effects of *H. sabdariffa* that prevent the progression of acute liver injury induced by PCM.

Histological Observation

In this study, the liver of the hepatoxicity-induced animals showed many picnotic nuclei (Fig. 1), suggesting that the liver cell were suffering from degeneration of the protein structure. In contrast, the liver of hepatoxicity-induced animals that were given *H. sabdariffa* showed a normal histology that was similar to the untreated animals (Fig.2). From these observations, it was suggested that *H. sabdariffa* might play a role in preventing further liver degeneration in the PCM-treated animals. In other studies, it was observed that the supplement of natural pigments of *H. sabdariffa* (anthocyanins) before the PCM treatment restored liver damage to normal as well (Ali *et al.*, 2003).

Interestingly, when given *H. sabdariffa* alone, the rats' liver showed no pathological changes in spite of the increases in the animals' GST, ALT and MDA levels. It is a relief that the liver of these rats showed normal morphology; however, an appropriate dose of *H. sabdariffa* needs to be determined so as to avoid any side effect, if

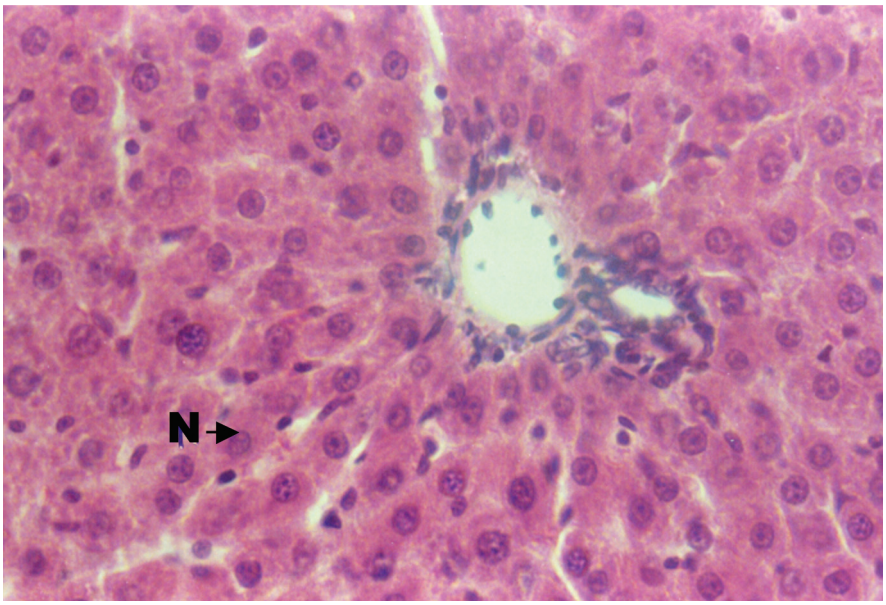

Fig.2: Liver of the untreated (control) animals stained with H & E (400x). The normal nucleus is indicated by the arrow. N = nucleus

the plant is to be consumed daily for a long period of time.

CONCLUSION

This study has shown that the post-treatment of *H. sabdariffa* aqueous extracts to PCM-induced hepatic toxicity rats prevents progression of acute liver injury by improving lipid peroxidation, reducing the level of GSH and increasing the level of GST and ALT caused by the PCM treatment. This preventive action could also be seen when the pathological changes of the liver in the PCM-treated animals were improved by giving them the post-treatment of *H. sabdariffa*. However, the consumption of unnecessarily high dosage of *H. sabdariffa* for long duration of time should be taken with caution.

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Using Factor Analysis to Distinguish between Effective and Ineffective Aggregate Stability Indices

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ABSTRACT

Several existing aggregate stability indices are commonly used to represent aggregate stability of soil. Consequently, there is a need to determine how well these common indices characterize or represent aggregate stability. The main objective of this study was to use a multivariate statistical method called factor analysis to determine the effectiveness of eight common indices in measuring aggregate stability. Eighty soil samples (Oxisols and Ultisols) were taken from soil depth of 0-150 mm and from different land uses, such as oil palm, coffee, tea, rubber, pine, fallow, vegetables, and grassland. Aggregate stability of these soils were determined by wet-sieving and water dispersion of the primary particles. Eight aggregate stability indices were used: AIA (average fraction of intact aggregates), WSA >0.3 and >0.5 (water-stable aggregates larger than size 0.3 and 0.5 mm, respectively), MWD (mean weight diameter), CR (clay ratio), WDC (water-dispersible clay), WDCS (water-dispersible clay plus silt), and TP (turbidity percentage). The factor analysis showed that all the aggregate stability indices were related to two common factors, namely, aggregate breakdown resistance and dispersion. By determining how well an aggregate stability index is correlated to either one or both these common factors, the factor analysis ranked the effectiveness of the indices as follows: WSA >0.3 = WDCS > AIA > MWD > WDC > CR. Due to the fact that WSA >0.5 is correlated very strongly with WSA >0.3, both the indices ought to be as effective as the other. The TP index, however, had a questionable efficacy as an aggregate stability index. Based on the findings of this study, it was therefore concluded that only two indices, WSA >0.3 (or WSA >0.5) and WDCS, were sufficient to represent the whole soil aggregate stability.

Keywords: Aggregate stability, factor analysis, Oxisols, structure, Ultisols, wet-sieving

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INTRODUCTION

Soil aggregate stability is the measure of the aggregates resistance to erosion caused by water or wind. There are several indices available to represent a soil's aggregate stability, but it is difficult to determine which index measures or represents aggregate stability better or the best. Currently, the relative effectiveness of several indices is gauged in two approaches.

The first approach is to correlate the aggregate stability indices between one another, as done by Ramos *et al.* (2003), Rohoskova and Valla (2004), and Nichols and Toro (2010). The idea is that if one could find an index that highly correlates with all other indices, it means this potential index is effective because it encompasses many aspects of aggregate stability, or this index can replace many indices. This idea appears sound, but in practice, it may not work. If all the other indices used for comparison are poor measures of aggregate stability themselves, then high correlations between them and the potential index only indicate that this particular potential index is the best among the worst indices. Even if widely accepted or established indices were used for comparisons, the correlations between them and the potential index are expected to be low or moderate, as noted by Epstein (1983). High correlations spell redundancy because the information provided by the potential index about aggregate stability is already provided by others. Low or moderate correlations are inconclusive because there is no way to tell merely from the correlations if the low or moderate correlations are because this potential index has provided information about aggregate stability unaccounted for by the other indices.

The second approach to test the effectiveness of several aggregate stability indices is to correlate them with the soil properties important to aggregate stability. This approach has been used by Albiach *et al.* (2001), Barthes and Roose (2002), Ramos *et al*. (2003), Li *et al.* (2010), and Nichols and Toro (2010). Using simple linear regressions or correlations, effective indices are ones that correlate highly to the soil properties. Again, this idea is sound, but the problem of this particular approach is that although the factors of aggregate stability are many, they may not all affect aggregate stability all the time and in all situations. Numerous researchers have shown that total organic matter may not always influence aggregate stability (Hamblin & Greenland, 1977; Dormaar, 1983; Albiach *et al.*, 2001). The same is also true for iron oxides (Deshpande *et al*., 1968). Moreover, these factors of aggregate stability can interact with one another; in other words, a factor may not, by itself, have a unique contribution to aggregate stability; instead, it jointly contributes, with another factor or factors, to affect aggregate stability. Such jointly contributions cannot be measured using simple linear regression or by correlations (Lapin, 1993).

The difficulties in determining which index is better or the best can be resolved by studying the proposal by Emerson (1954), and Emerson and Greenland

(1990). They noted that, ultimately, the disruption of aggregates is by two ways, i.e., either by breaking them down into smaller aggregates (slaking), or by discharging their primary particles (dispersion) (*see* Fig.1). As aggregate stability is the measure of the aggregates' resistance to disruption, aggregate stability then encompasses these two subsets, namely, slaking and dispersion.

Whichever measurements of aggregate stability are used, they must ultimately relate back to either or both of the slaking or dispersion phenomena. This insight is crucial because it suggests a way to assess the effectiveness and interrelationship among the various aggregate stability measurements based on how well they relate back to these two aggregate breakdown phenomena.

Fig.2 shows a conceptual model that relates six measurement methods to the two aggregate breakdown phenomena; where y_i is the i-th measurement method; ε_i is the measurement error for the i-th measurement method; η_i is the i-th breakdown phenomenon where $i = 1$ denotes slaking, and $i = 2$ denotes dispersion; and $\lambda_{1,i}$ and $\lambda_{2,i}$ are the coefficients representing the effect of η_1 (slaking) and η_2 (dispersion), respectively, on y_i. Finally, $\rho_{1,2}$ is the correlation between slaking and dispersion. The model can be described in a linear form by:

$$y_i = \lambda_{1,i}\eta_1 + \varepsilon_i \qquad \text{for } i = 1, 2, \text{ and } 3$$

where these measurements are related to slaking, and

$$y_i = \lambda_{2,i}\eta_2 + \varepsilon_i \qquad \text{for } i = 4, 5, \text{ and } 6$$

for measurements related to dispersion.

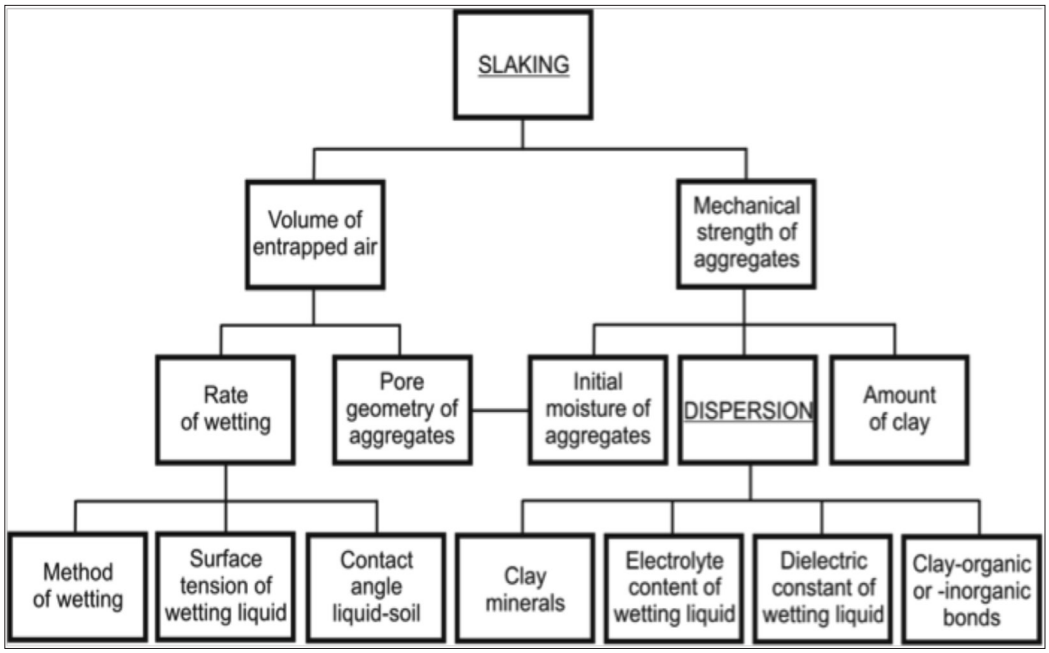

Fig.1: Important factors of slaking and dispersion (after Emerson, 1954)

TABLE 1
The range of the mean particle size distribution for the soils used in this study

Soil taxonomy	Land use	%clay <2 μm	%silt 2-50 μm	%sand >50 μm
Typic Paleudult	Oil palm	8.3 – 34.7	16.7 – 71.4	12.8 – 59.2
Typic Hapludox	Coffee	21.7 – 70.1	7.3 – 29.2	21.6 – 49.1
Typic Paleudult	Fallow	42.3 – 67.7	9.2 – 21.0	22.0 – 36.7
Typic Paleudult	Tea	35.7 – 53.0	15.5 – 17.6	30.5 – 48.5
Typic Paleudult	Vegetables	55.4 – 60.1	5.6 – 7.7	32.7 – 38.1
Xanthic Hapludox	Pine	33.4 – 42.7	19.2 – 21.0	38.6 – 47.0
Typic Paleudult	Rubber	20.7 – 41.6	18.0 – 36.2	22.1 – 61.4
Typic Paleudult	Grassland	43.0 – 51.0	16.0 – 20.6	29.2 – 40.9

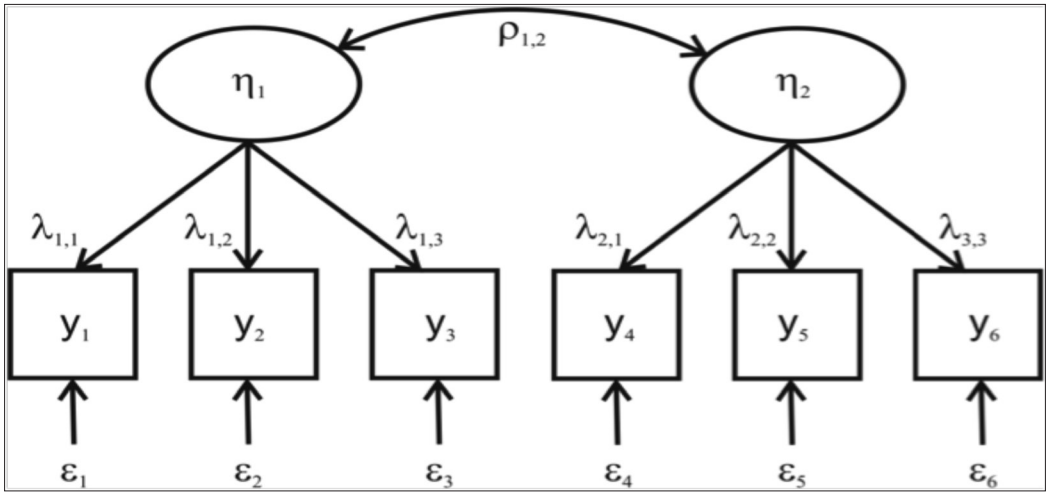

Fig.2: Conceptual model of aggregate stability measurements

Thus, it is possible to determine the effectiveness of each measurement on aggregate stability by determining the value of each λ because its magnitude tells how well a measurement actually measures the real value of aggregate stability. The best measurement is the one with the highest λ for the respective breakdown phenomenon, η. The various values of λ and the correlation between slaking and dispersion can be determined using a multivariate statistical method known as the factor analysis (Brown, 2006). Applied in the context of aggregate stability, the factor analysis will reveal if the various measurements of aggregate stability have two common factors (namely, slaking and dispersion), and the degree each measurement measured slaking and dispersion.

Consequently, the main objective of this study was to use the factor analysis to determine the effectiveness of several

established indices as a measure of whole soil aggregate stability.

MATERIALS AND METHODS

Ultisol and Oxisol soils (Table 1) from eight land use areas were sampled from Universiti Putra Malaysia. The land uses included oil palm, coffee, tea, rubber, pine, fallow, vegetables, and grassland. From each land area, ten soil samples were sampled randomly in the field, and the sampling was done from 0-150 mm soil depth using a soil auger. It is important to note that only one soil sample was taken from each sampling point. The soil depth 0-150 mm was selected as the sampling depth because the aggregate stability between the soils is mostly different from one another in the top soil layer compared to the lower or sub-soil layers.

Thus, eighty soil samples were air-dried for at least one week prior to the analysis. The particle size distributions for the soils are shown in Table 1. Before any aggregate stability tests, all the soil samples were pre-wetted by incubation under room temperature and at approximately 98% relative humidity for 24 hours. Meanwhile, the analyses of each soil sample were done in triplicates.

Particle size distribution was analyzed by the pipette method (Gee & Bauder, 1986), and the percentages of the primary particles were used to calculate the clay ratio index (CR) (Bouyoucos, 1935) as follows:

$$CR = \frac{\%\text{sand} + \%\text{silt}}{\%\text{clay}}$$

Wet-sieving was done according to the method proposed by Kemper and Chepil (1965). The samples were dry-sieved using a nest of sieves with openings of 8.0, 5.0, 3.0, 2.0, 1.0, 0.5 and 0.3 mm. Wet-sieving was for 30 minutes, at 40 strokes per minute, and through a vertical distance of 4.0 cm. After wet-sieving, the aggregates retained in each sieve were separately collected, oven-dried, and weighed. After weighing, the sand content in each aggregate size fraction was determined for sand correction calculations. From wet-sieving, four aggregate stability indices were calculated: AIA (average intact aggregates), WSA >0.5 (water-stable aggregates above the size of 0.5 mm), WSA >0.3 (water-stable aggregates above the size 0.3 mm), and MWD (mean weight diameter).

The AIA index (in percent) expresses the average fraction of the aggregates that remained intact (i.e., did not breakdown into smaller pieces) after wet-sieving. It was calculated by:

$$AIA = \frac{100}{N} \times \sum_{i=1}^{N} \frac{W_{a,i} - W_{b,i} - S_i}{W_{a,i} - S_i}$$

where $W_{a,i}$ and $W_{b,i}$ are the weight of aggregates size fraction i before and after wet-sieving, respectively (i = 1 to N), N is the number of aggregate size fractions, and s_i is the weight of sand in aggregate size fraction i. The indices WSA >0.5 and WSA >0.3 (both in percent) were calculated by:

$$WSA > 0.5 = 100 \times \frac{\text{weight of agg.} > 0.5\text{mm} - \text{weight of sand} > 0.5\text{ mm}}{100 - \text{weight of sand} > 0.5\text{ mm}}$$

$$WSA > 0.3 = 100 \times \frac{\text{weight of agg.} > 0.3\text{mm} - \text{weight of sand} > 0.3\text{ mm}}{100 - \text{weight of sand} > 0.3\text{ mm}}$$

where the value 100 in both denominators was the total weight of soil (100 g) used for wet-sieving. The index MWD (mm) expresses a given soil's mean weight diameter after wet-sieving (van Bavel, 1953). It was calculated by:

$$MWD = \sum_{i=1}^{N} \frac{W_{b,i}}{100} \times \bar{x}_i$$

where $\bar{x}_i$ is the mean diameter of the aggregates in the size fraction, i; and the value 100 in the denominator, like before, is the total weight of soil (100 g) used for wet-sieving.

Lastly, three more indices, WDC (water-dispersible clay), WDCS (water-dispersible clay plus silt) and TP (turbidity percentage), were used. To calculate WDC and WDCS, the method of Soil Survey Laboratory Staff (1992) was followed. Five grams of uncrushed soil (<2 mm) was added into 50 ml distilled water (ratio soil to water was 1:10), and an end-over-end shaking was for 30 minutes and at 40 rpm. The contents were then poured into a 1-liter measuring cylinder; the volume made up to one litre, the solution gently stroked up-down to distribute the contents, and then left for four minutes for the undispersed aggregates and sand particles to settle to the bottom. The clay and silt particles were then siphoned off at 10 cm depth using a 25 ml pipette. At an appropriate settling time, the clay particles were siphoned off at 10 cm depth using a 25 ml pipette. These values were used to calculate the indices WDC and WDCS (both in percentage) as:

$$WDC = 100 \times \frac{\%\text{dispersed clay}}{\%\text{clay (from particle size analysis)}}$$

$$WDCS = 100 \times \frac{\%\text{dispersed clay and silt}}{\%\text{clay and silt (from particle size analysis)}}$$

The index TP (in percentage) was calculated based on the turbidimetric method of Williams *et al.* (1966). Two grams of soil (< 2 mm) was added with 20 ml distilled water (1:10) and shaken end-over-end for 30 minutes and at 40 rpm. Another 2 g of the same soil sample (< 2 mm) was added with 20 ml Calgon (sodium hexametaphosphate) and shaken end-over-end for 15-16 hours and at 80 rpm. After shaking, both the solutions were left to settle for 4 minutes, and this was followed by pipetting 1 ml out of each solution. To each of those 1 ml solutions, 24 ml of distilled water was added, mixed, and their turbidities were immediately read using a turbidity meter (ELE Paqualab, ELE International, Hertfordshire, England). The TP index was calculated by

$$TP = 100 \times \frac{\text{turbidity of dispersed sample (in water)}}{\text{turbidity of maximum dispersion (in Calgon)}}$$

The factor analysis was used to identify the structure within the set of aggregate stability indices. Before the factor analysis was used, the data set was tested to determine whether it was appropriate for the factor analysis. For this purpose, two statistical tests of factor analysis appropriateness, Bartlett's (1951) Test of Sphericity and Kaiser-Meyer-Olkin (KMO) measure of

TABLE 2
Correlation matrix between all pairs of the aggregate stability indices

	AIA	MWD	WSA >0.5	WSA >0.3	WDC	WDCS	TP
MWD	0.77**	-					
WSA>0.5	0.91**	0.86**	-				
WSA>0.3	0.87**	0.80**	0.98**	-			
WDC	-0.45**	0.26*	-0.45**	-0.47**	-		
WDCS	-0.63**	-.041**	-0.66**	-0.69**	0.77**	-	
TP	0.15	0.23*	0.34**	0.43**	-0.10	-0.10	-
CR	-0.63**	-0.61**	-0.77**	-.076**	0.37**	0.53**	-0.34**

* $p < 0.05$; ** $p < 0.01$

sampling adequacy, were used (Tobias & Carlson, 1969). The factors were extracted by Principal factor extraction method, while the number of factors was selected based upon Cattell's Scree test, and the rotation of the factors was done using oblique rotation by Direct Oblimin method (Brown, 2006). All the factor analysis computations were done using SPSS for Windows version 16 (SPSS Inc., Chicago).

RESULTS

The interrelationships between the eight aggregate stability indices were determined using the factor analysis. However, before any analysis, the indices were checked for violations of normality. Only the clay ratio (CR) index showed violation of normality (skewness=2.71; kurtosis=7.79), and was transformed by ln(CR×100).

All indices generally showed moderate to strong correlations with one another (Table 2). Meanwhile, WSA >0.5 was found to strongly correlate with WSA >0.3 (r=0.98**). In addition, both of these indices generally correlated the highest with all the other indices. The correlation between each of these indices with MWD was strong (r=0.86** for WSA >0.5 and r=0.80** for WSA >0.3); however, this was not as strong as AIA (r=0.91** for WSA >0.5 and r=0.87** for WSA >0.3). Compared with MWD, the AIA index had stronger correlations with the other indices.

Based on the strength of the correlation coefficients, there were generally three groups of indices. The first group comprised the AIA, MWD, WSA>0.5, and WSA >0.3 indices. These indices had stronger correlations between themselves than their correlations with the indices in the second group of indices: WDC, WDCS, and CR. The indices in the second group, however, correlated only moderately between themselves.

The third group of indices actually comprised of only a single index, i.e. TP. This sole index correlated poorly with almost all of the other indices. Nonetheless, TP correlated positively with the indices in the first group (AIA, MWD, WSA >0.5,

TABLE 3
Principal component transformation to determine the relationship between the TP index with the rest of the other indices

Indices	Component 1	Component 2	Component 3
WSA >0.3	**0.94**	-0.59	-0.43
AIA	**0.92**	-0.54	-0.13
MWD_W	**0.91**	-0.28	-0.21
CR	-0.80	0.46	0.43
WDC	-0.36	**0.95**	0.12
WDCS	-0.59	**0.93**	0.12
TP	0.27	-0.11	**-0.99**

and WSA >0.3), but negatively with the indices in the second group (CR, WDC, and WDCS).

The method by Flury and Riedwyl (1988) was followed to determine if TP possessed a questionable efficacy as an aggregate stability index. The principal component analysis, a variant of the factor analysis, was used to explain as much variance among the indices as possible, i.e. to represent the relationship patterns in the correlation matrix to fewer components so that the interrelationships among the indices could become clearer. For the subsequent analyses, WSA >0.5 was disregarded as it could be represented by WSA >0.3 because of their high, almost perfect, correlation between each other. Three components were extracted based upon the Scree test and rotation was by the Direct Oblimin method.

Table 3 shows the results of the principal component transformation (component extraction and rotation) of the indices. It shows that TP revealed a questionable efficacy as an aggregate stability index. In fact, TP almost entirely defined the third component. It highly correlated with the third component (r=-0.99), whereas, the other indices insignificantly correlated with the same third component. The first and second components moderately to strongly correlated with all the indices, except with TP. Moreover, the correlation matrix between the third component and the indices (*see* Table 3) resembled the correlation matrix between TP and each of the other indices, as previously shown in Table 2. Thus, TP appeared to be the third component itself, i.e. a separate "entity" from the rest, representing a different concept other than aggregate stability. In a preliminary analysis, including TP into the factor analysis was found to have reduced the reliability of the factor analysis model. For example, including the TP index into the factor analysis was shown to reduce the KMO sampling adequacy and the total variance accounted for by the factor model. Therefore, the TP index was discarded from the subsequent analyses.

As for the factor analysis, the following six indices were used: AIA, MWD, WSA >0.3, WDC, WDCS, and CR. Prior to the analysis, all the indices were standardized to

TABLE 4
Correlation of the common factors with the aggregate stability indices; (a) unrotated factor structure, and (b) rotated factor structure

(a) unrotated factor structure

Indices	Factor 1	Factor 2	Variance explained by the factors
WSA >0.3	0.96	0.19	0.97
AIA	0.88	0.17	0.80
WDCS	-0.82	0.54	0.97
MWD	0.77	0.41	0.76
CR	-0.73	-0.14	0.55
WDC	-0.60	0.51	0.62
Variance explained by the indices	0.64	0.13	0.78

(b) rotated factor structure

Indices	Factor 1	Factor 2
WSA >0.3	**0.98**	-0.63
AIA	**0.89**	-0.57
MWD	**0.86**	-0.34
CR	**-0.74**	0.48
WDCS	-0.61	**0.98**
WDC	-0.42	**0.79**

have zero means and variances of one. The appropriateness of the data was tested and found to be suitable for the factor analysis because of the following: (1) Bartlett's Test of Sphericity was convincingly rejected (389.99; $p<0.0001$), and (2) KMO sampling adequacy was measured at 0.8 (1.0 being the highest). At this KMO measure, the appropriateness of the data for the factor analysis was rated as "meritorious", i.e. one rank lower than the highest rating (Kaiser & Rice, 1974). Moreover, the factor analysis produced a low anti-image covariance matrix and reproduced the correlation matrix (as shown in Table 2) accurately with no residuals having absolute values above 0.05. These validation results indicated that using the factor analysis was appropriate to determine the internal structure of these six indices. Extraction of factors was done through the Principal factor method, while rotation was by the Direct Oblimin method. Two common factors were selected based on the Scree test. The results gathered from the factor analysis are shown in Table 4.

The data presented in Table 4 show that the six aggregate stability indices, though different from one another, were related to one another by two common factors. In other words, the six indices were ultimately related to two general aspects of aggregate stability—as represented by the two common factors.

To identify the first and second common factors, it is important to consider the proposal by Emerson (1954), as well as Emerson and Greenland (1990), shown in Fig.1. As mentioned previously, the researchers noted that the aggregate breakdown encompasses only two main phenomena, namely, slaking and dispersion. Slaking is the breakdown of the aggregates due to explosion of entrapped air within the aggregates, whereas, dispersion is the discharge of the primary particles from the aggregates. It is crucial to highlight that slaking is usually measured using the wet-sieving method.

From the factor structure in Table 4b, the first common factor correlated strongly with the first three indices; namely, AIA, MWD, and WSA >0.3. These three indices are so-called the "wet-sieving indices" because they were derived from the results of the wet-sieving process. In addition, the three indices tended to measure the ability of the aggregates to retain their sizes during the disruptive effects of water. On the other hand, the second common factor correlated more strongly with the “dispersibility indices” that were derived from the dispersion of clay and silt particles. These indices were WDC and WDCS.

Based on the proposal by Emerson (1954), and Emerson and Greenland (1990), the first common factor could therefore be interpreted as slaking, while the second common factor as dispersibility. Although the first common factor correctly represents slaking, it is an imprecise description of how aggregates breakdown. Slaking is only one way larger aggregates could breakdown into smaller pieces. Other physical disruptions, such as by water agitation during wet-sieving or the falling impacts of raindrops, can also cause aggregate breakdown. Therefore, it would be more precise to interpret the first common factor as representing a larger, more generic aspect than slaking. Thus, the first common factor was interpreted as representing the aggregate breakdown resistance, while the second common factor remained as the dispersion aspect.

While the data in Table 4b helped to identify the two common factors, those in Table 4a were used to determine the effectiveness of the indices. The main criterion to determine the effectiveness of an index is to determine the proportion of its variance involved in the measurement of aggregate stability. The data in Table 4a revealed that WSA >0.3 and WDCS were the two most effective indices of aggregate stability. This was because 97% of the variance in WSA >0.3 and in WDCS could respectively be explained by the two common factors; that is, only a mere 3% of their variance was not involved in the measurement of aggregate stability. The least effective index was CR because only about half of its variance could be explained by the two common factors. Thus, the effectiveness of indices could be ranked as follows: WSA>0.3 = WDCS > AIA > MWD > WDC > CR.

Although WSA >0.3 and WDCS were equally the most effective indices, their measurement emphasis on aggregate stability was different from each other. WSA

>0.3 measured the aggregate breakdown resistance very strongly (r=0.96) but almost not measuring dispersion at all (r=0.19). WDCS, on the other hand, measured both aggregate breakdown resistance (r=-0.82) and dispersion (r=0.54). For aggregate breakdown resistance, WSA >0.3 not only measured this aspect more effectively than WDCS, it this was also done more effectively than any other indices. For dispersion, however, WDCS clearly measured the second aspect of aggregate stability more effectively than WSA >0.3, as well as measuring dispersion the highest as compared to the other indices. Tables 4a and b show that no index measures aggregate breakdown resistance and dispersibility equally well.

This also means that to measure aggregate stability more effectively, only two indices (WSA >0.3 and WDCS) are sufficient. In this way, both the aspects of aggregate stability would be measured: WSA >0.3 stressing very strongly on the aggregate breakdown resistance aspect, and WDCS index is needed to include or measure the dispersion aspect.

The factor model could explain 78% of the variance in all the six indices (*see* Table 4a). All the six indices could explain 64% of the variance in the aggregate breakdown resistance. In addition, 13% of the variance in dispersibility was explained by all six indices. This imbalanced proportion indicated that the six indices measured the breakdown resistance of the aggregates more than dispersibility. This is true for every index.

Finally, the factor analysis showed that the correlation coefficient between the two common factors was -0.55, suggesting that the aggregate breakdown resistance and dispersion shared a moderate and inverse relationship with each other, and both shared approximately 30% of the variance.

DISCUSSION

The factor analysis has showed that no matter how different the aggregate stability indices are from each another, or what aspects of aggregate stability they measure or emphasize, all the indices have been found to ultimately relate to either or both of the aggregate stability phenomena; aggregate breakdown resistance and dispersibility. These phenomena were slightly modified from what Emerson (1954) and Emerson and Greenland (1990) had earlier proposed (Fig.1). The researchers further remark that aggregate stability encompasses two main aspects, namely, slaking and dispersion. However, to narrow the first main aspect of aggregate stability to slaking is imprecise. This is because, aggregates can also breakdown into smaller aggregates by the destructive forces from water agitation or the falling impact of raindrops, apart from slaking. Therefore, it would be more precise to represent the first aggregate stability aspect as aggregate breakdown resistance rather than merely slaking.

Thus, the factor analysis provides a way to distinguish effective indices, which include those that correlate strongly to either one, or both aggregate breakdown resistance and slaking. On the contrary, any index that

fails to correlate strongly to at least one of these phenomena has a doubtful efficacy, such as the TP index, as revealed in this study.

In this study, the effectiveness of the six indices could be ranked as follows: WSA >0.3 = WDCS > AIA > MWD > WDC > CR. Due to its strong correlation with WSA >0.3, WSA >0.5 would just be as effective as WSA >0.3. The factor analysis has also been shown to measure aggregate stability effectively on a whole, and only two indices (WSA >0.3, or WSA >0.5 and WDCS) are needed for the purpose. In this way, both the aspects of aggregate stability would be measured: WSA >0.3 (or WSA >0.5), stressing very strongly on the aggregate breakdown resistance aspect, and the WDCS index is needed to include or measure the dispersion aspect. However, if ease and speed of measurement are crucial, WDCS is recommended since it measures aggregate breakdown resistance effectively (although it is not as effective as WSA >0.3 or WSA >0.5), and at the same time, measuring dispersion moderately well. Correspondingly, this kind of dual measuring effectiveness shows that no index measures aggregate breakdown resistance and dispersibility equally well. Thus, an aggregate stability index "specializes" only on one aspect.

The high effectiveness of WSA >0.3 and WSA >0.5 challenges the warning as noted by some researchers that using stability greater than a single size fraction is inaccurate. For example, Low (1954) discovered that the percentage of water-stable aggregates between 0.25 and 1 mm decreased, whilst those greater than 3 mm were found to increase. If a single fraction of aggregates greater than 0.25 mm was used, it would have indicated that aggregate stability did not change. This implies that indices like WSA >0.3 and WSA >0.5 are insensitive to changes in the stability of a given aggregate size fraction. Moreover, using such indices means the researcher tolerates the breakdown of larger aggregates more than the breakdown of smaller aggregates. This is particularly because to pass through a 0.5 mm sieve, for instance, the aggregates in the size 8 mm must breakdown several times or breakdown more than the aggregates of size 1 mm. All the above points are valid but only to some degree, because these points assume that the aggregates from one size fraction behave independently from those in other size fraction. Although the stability of one aggregate size fraction may be different from another, they nevertheless share some soil characteristics that cause various aggregate size fractions to be related (Kemper & Rosenau, 1986; Loveland & Webb, 2003). This means, if the stability of an aggregate size fraction is weak, the stability of other aggregate size fractions would be weak as well. Such close dependencies between the various aggregate size fractions may explain why WSA >0.3 and WSA >0.5 were not affected by the above points.

On the other hand, the commonly used MWD was an ineffective aggregate stability index. Part of the problem is the arbitrary weights assigned to each aggregate size

fraction. What MWD actually represents is the weighted average size of the aggregates produced after wet-sieving. The weight assigned to an aggregate size fraction is the average diameter of all the aggregates in that size fraction. However, these weights are arbitrary because there is no proof that, in equal weight, aggregates of 8 mm are always two times more stable than those of 4 mm, even though a specific weight of 8-mm aggregates suggests greater stability than an equal weight of 4-mm aggregates (but this does not necessarily mean two times greater stability). Another problem with MWD is that the various proportions of all the aggregate size fractions are averaged without sand correction. Without such correction, loose or unbounded sand particles are falsely regarded as aggregates. As the soils used in this study are varied widely in their sand amount, sand correction is therefore vital to avoid this fallacy.

The indices AIA and MWD did not measure aggregate stability as well as WSA >0.3 or WDCS, and this is probably because AIA and MWD are the mean values of several proportions. Averaging the various proportions is a crude representation because averaging is sensitive to the distribution of the various proportions. For example, Swift (1991) observed that a single value of MWD used in his study was not the mean aggregate stability of a uniformly grouped normal or Gaussian distribution of aggregate stability values, but it was the mean of widely spaced values with significantly large numbers of values grouped at the extremes of the distribution range. Swift also remarked that using MWD was not suitable, and that it would be better if aggregate stability was observed by comparing the most stable with the least stable aggregates.

Factor analysis also revealed that the indices of aggregate stability tended to emphasize more on the ability of the aggregates to resist breakdown and less on dispersibility. The reason for this is shown in Fig.1. This chart shows that slaking (or aggregate breakdown) is a broader aspect than dispersion, being influenced by more factors, and that dispersion is a subset of slaking. From Fig.1, the factors important to dispersion (such as the characteristics of the liquid and the type of clay minerals) are similar in all the soil types used in this study. Although the soils were not analyzed for their clay mineral types, it is unlikely that these soils (Ultisols and Oxisols) would have such differing clay mineral types to affect dispersibility differently. The only important factor affecting dispersibility differently between the soils is the amount (and type) of organic and inorganic compounds that bind the clay particles (Chenu *et al.*, 2000; Boix-Fayos *et al.*, 2001; Six *et al.*, 2004; Noellemeyer *et al.*, 2008).

Slaking phenomena, on the other hand, is influenced by the same factors affecting dispersion and by other factors unique only to slaking. All this means that slaking is influenced by more extensive factors than dispersion, and why slaking (hence, also aggregate breakdown resistance) tends to be stressed more by the aggregate stability indices as compared to dispersion. In

this study, slaking was stressed by the indices approximately five times more than dispersion.

Because dispersion is a subset of slaking, the relationship between the two ought to be at least moderately close. The correlation coefficient between aggregate breakdown resistance and dispersion, as shown by factor analysis, was -0.55, or both factors sharing approximately 30% of variance. This is an expected relationship because the soils that disperse easily ought to breakdown easily as well.

Clay ratio (CR) was shown to be the second worst index of aggregate stability (the worst index was TP). This index CR ignores the level or state of soil structure, and it only takes into account the particle size distribution of the soil. The particle size distribution, though important, would only explain or affect aggregate stability partially; therefore, the correlation of CR to aggregate stability is rather low. As shown in Fig.1, the amount of clay is an important factor not to dispersion but to slaking. This is why, as shown by the factor analysis, CR is correlated more to the first common factor than to the second common factor.

On the other hand, the TP index was the worst and a questionable aggregate stability index. Turbidmetric methods are useful for comparing treatments of the same or similar soils types, but they are unsuitable for comparing the types of soil with different particle size distributions (Douglas & Goss, 1982). In this study, the poor reliability of TP was probably due to two other factors. First, the soils used in this study varied in their colours, ranging from yellow to yellowish brown to brown. These colour variations may have complicated the turbidity comparisons between the soils. Second, in this study, before the turbidities of samples were read, the dispersed soil solutions were diluted 25 times. This was necessary to standardize the soil:water ratio to 1:10 because this particular ratio was also used to measure the dispersibility of the soils, as measured by WDC and WDCS. In keeping to this ratio, however, the turbidities of the dispersed soil solution was too high to be read by the turbidity meter and thus, it had to be further diluted. The error variation caused by these dilutions may have been too large.

The factor analysis is a powerful tool because it determines the internal relationship structure of the various indices. The factor analysis untangles and summarizes the relationship patterns among the indices so that the indices' relationships among each other and to aggregate breakdown resistance and dispersion can be determined.

CONCLUSIONS

The factor analysis has shown that no matter how different the indices are from each other, or which aspects of aggregate stability the indices measure, all the indices are related to two main aspects of aggregate stability, namely, aggregate breakdown resistance and dispersion. By determining how well an aggregate stability index is correlated to either one or both aggregate breakdown resistance and dispersion, the factor analysis ranked the effectiveness of

the indices as follows: WSA >0.3 = WDCS > AIA > MWD > WDC > CR. Thus, it could be concluded that only two indices were sufficient to represent the whole soil aggregate stability effectively, namely WSA >0.3 and WDCS.

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Evidence of Diazotrophic Symbionts in the Leguminous Cover Crop *Mucuna bracteata*

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ABSTRACT

New studies point to an increasing number of identified bacteria that can nodulate and fix N_2 in legumes which do not belong to the original genus of *Rhizobium* and the *rhizobial phylogenetic* lineages. This study was conducted to isolate and identify diazotrophic microsymbionts from the root nodules of *Mucuna bracteata* (an important cover crop for oil palm) based on nitrogenase gene (*nifH*) isolation and partial 16S rDNA sequence analysis. The findings of this study indicated that the isolated microsymbionts could nodulate and promote N_2-fixation activity in *M. bracteata*. These also contributed to enhanced plant growth in terms of leaf protein and chlorophyll content, as well as in the biomass of whole plants and nodules. Additionally, *nifH* gene fragments were successfully amplified at ~380 bp from eight of the isolates (USM accessions A11, B4, B9, B12, B19, C1, C4 and C8) using *nifH*3 primers, while the remaining isolates (namely, USM accessions B14, B15, B20, C2 and C9) were successfully amplified at various sizes (~550, 650, 350, 450, and 900 bp, respectively) using *nifH*4 primers. The partial 16S rDNA sequencing revealed that the diazotrophic microsymbionts were not only from the traditional *Alphaproteobacteria* class *(Brevundimonas sp.*), but also from the *Betaproteobacteria* class (*Achromobacter* sp. and *Burkholderia sp.*) and the *Gammaproteobacteria* class (*Stenotrophomonas* sp.). Five non-rhizobial isolates were obtained and identified as *Bacillus* sp. from the root nodules of *M. bracteata.* The findings indicate the diversity of potentially-beneficial diazotrophic microsymbionts active in this emerging legume species.

Keywords: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Mucuna bracteata, Diazotrophic microsymbionts

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INTRODUCTION

Rhizobia can infect the roots of leguminous plants, leading to the formation of nodules

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wherein nitrogen (N_2) fixation takes place. This symbiosis plays a very important role in agriculture as it can relieve the requirements for nitrogenous fertilizers during the growth of leguminous crops. The term 'rhizobia' has been used for all the bacteria that are able to produce nodules and fix atmospheric nitrogen in legumes (Brewin, 2004; Cheng, 2008). Traditionally, rhizobia were exclusively members of the *Rhizobiaceae* family in the *Alphaproteobacteria* class of bacteria, which includes the genera *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium* (Sprent, 2001; Sawada *et al*., 2003).

New studies, however, have shown the ability of many diazotrophs to nodulate and fix N_2 in legumes which do not belong to *Rhizobium* in the *Alphaproteobacteria* class (Willems, 2006). These diazotrophs include other species within the *Alphaproteobacteria* class (e.g., *Methylobacterium* and *Devosia*), as well as *Burkholderia* and *Ralstonia* in the *Betaproteobacteria* class. Numerous species of *Betaproteobacteria* have recently been isolated from the root nodules of leguminous plants (Chen *et al*., 2005). For example, the strains of *Burkholderia* have been isolated from a variety of legumes such as *Mimosa* spp. (Chen *et al*., 2005; Pandey *et al*., 2005; Elliott *et al*., 2007b) and two papilionoid species (*Macroptilium atropurpureum* and *Cyclopia* spp.) (Elliott *et al*., 2007a). In addition, *Stenotrophomonas maltophilia* (in the *Gammaproteobacteria* class) has also been shown to nodulate legumes (Kan *et al*., 2007). However, more knowledge is needed regarding the diversity and N_2-fixing ability of these bacteria in emerging leguminous cover crops.

The current taxonomy has revealed a wide diversity of diazotrophic microsymbionts that are able to form N_2-fixing symbioses with legume roots in a manner that is similar to rhizobia at the genus, species and intraspecies level. However, relatively little information is available regarding diazotrophic species associated with the leguminous cover crop *Mucuna bracteata*, an emerging cover crop for plantation production in tropical Asia. Thus, the objectives of this study were: 1) to determine indigenous microsymbiont strains which could further promote symbiotic N_2-fixation activities for *M. bracteata*, and 2) to verify the identity of the isolated microsymbionts from *M. bracteata,* based on their partial 16S rDNA sequences.

MATERIALS AND METHODS

Isolation of Diazotrophic Microsymbionts from Root Nodules

The nodulated roots of mature *M. bracteata* plants were collected randomly from Taiping Rubber Plantation, Perak, Malaysia. Then, fresh nodules from the roots were detached and preserved in universal bottles containing desiccant (silica gel) and cotton wool for later analysis. The colonies of microsymbionts were isolated from the nodules via a standard laboratory methodology described in Somasegaran and Hoben (1985). Purity of the strains was ensured by single colony isolation, observation of colony morphology on Yeast

Extract Mannitol Agar (YEMA) containing Bromothymol Blue (BTB) and Red Congo (RC) indicators (Yang *et al*., 2008), and by Gram staining (Vincent, 1970, 1982; Somasegaran & Hoben, 1985). The colonies of the pure cultures were maintained on YEMA slants at 4°C and also stored for later use in 15% (v/v) glycerol at -20°C.

Nodulation and N_2-fixation Screening of Diazotrophic Microsymbionts in M. bracteata

The isolated microsymbionts were cultured in YEM broth (YEMB) and shaken at 100 rpm for 3 days for fast growers and 5 days for slow growers, respectively (Vargas-Ayala *et al*., 2000). Simultaneously, the seeds of *M. bracteata* were surface-sterilized using 95% (v/v) ethanol, 0.1% (v/v) mercuric chloride ($HgCl_2$) solution, washed 5 times with sterile distilled water and germinated aseptically for 3-4 days in the dark (Somasegaran & Hoben, 1985). The seedlings were sown in pots containing 1 kg sterilized sand (to allow maximal air flow through the roots for H_2 evolution analysis) and were inoculated with 5 ml (10^9 ml^{-1}) of the respective isolates at D_0, D_{20} and D_{40}.

The experiment was laid out in a completely randomised design (CRD) with each treatment consisting of four replicates. The respective treatments for the plants consisted of: (1) Control 1 (uninoculated plants receiving fertilizer containing N (0.05 M KNO_3)); (2) control 2 plants (uninoculated plants receiving N-free fertilizer); and (3) inoculated plants (inoculated with locally isolated microsymbionts and received N-free fertilizer). The plants were maintained in the green house for 65 days of growth (D_{65}) before harvesting. Controls 1 and 2 were included to determine the effects of fertilizer compared to the inoculated microsymbionts. The seedlings were watered daily with N-free nutrient solution as recommended by Hunt and Layzell (1993). A week prior to the harvest day, H_2 evolution tests were conducted to measure the N_2-fixation activity of the inoculated host plants by using a gas flow system fabricated by Qubit Systems (Logger Pro 3.2; Kingston, Ontario, Canada) (Hunt & Layzell, 1993; Curtis *et al*., 2004). The system includes an AC gas pump, a gas bag containing Ar:O_2 (80:20), a flow meter, a desiccator column filled with fresh magnesium perchlorate ($Mg(ClO_4)_2$), a hydrogen gas sensor and a Vernier LabPro interface (Beaverton, Oregon, USA). Pots with the inoculated plants were sealed properly and attached to the gas exchange system. Air was pumped through the pot and was controlled by the flow meter. H_2 production was detected continuously by the H_2 sensor in the system which is linked to the computer. Ar:O_2 was used as the indicator to measure total electron flux through nitrogenase in the H_2 evolution rate assay. The N_2 fixation rate was calculated from the rate of H_2 evolution as described by Qubit Systems (Layzell *et al*., 1984, 1989; Hunt & Layzell, 1993; Moloney *et al*., 1994; Curtis *et al*., 2004). At harvest (D_{65}), the plants were analyzed for total length, number of leaves, leaf chlorophyll and protein contents (Lowry *et al*., 1951), number of nodules, dry weight of nodules

and plant biomass (Houngnandan *et al*., 2001). The data were statistically analyzed via one way Analysis of Variance (ANOVA) using SPSS V 15.0 software. The Tukey procedure, $p<0.05$ was chosen to test the significant differences between the means (Colman & Pulford, 2006).

DNA Extraction

Genomic DNA was extracted from the bacterial cultures grown in Luria-Bertani broth. For this study, i-genomic CTB DNA extraction mini kits from iNtRON Biotechnology (Seongnam, South Korea) were used essentially to extract the DNA. The extracted genomic DNA was quantified at $OD_{260/280}$ via UV spectrophotometer (GeneQuant pro; Amersham Biosciences/ GE Healthcare, Uppsala, Sweden).

PCR Amplification and Sequencing of nifH *Gene Fragments*

In order to amplify *nifH* gene fragments, two sets of *nifH* degenerate oligonucleotides were used: 1) *nifH*3 forward primer (5'-TAY GGN AAR GGN GGN ATN GGN AA-3') with nifH3 reverse primer (5'-GCR AAN CCN CCR CAN ACN ACR TC-3') (Choo *et al*., 2003); and 2) *nifH*4 forward primer (5'-TAY GGI AAR GGI GGI ATI GGI AA-3') with *nifH*4 reverse primer (5'-GCR AAI CCI CCR CAI AG ACR-3'). Primer *nifH*4 was designed based on primer *nifH*3 by replacing the degenerate nucleotide N with I to increase the accuracy of the primer. A 50 µl sample of the PCR reaction mixture was prepared and it contained the template genomic DNA (80 ng μl^{-1}) in 10x PCR buffer, 1.5 mM $MgCl_2$, 25 pmoles of each primer, 0.2 mM of each dNTP and 1U of Taq DNA polymerase. PCR amplifications were carried out with a Bio-Rad thermocycler (Hercules, California, USA) in the following conditions and with slight modifications (Choo *et al*., 2003): an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s each, annealing at 45°C for 30 s, extension at 72°C for 30 s and a final extension step at 72°C for 10 min. Meanwhile, amplification of the *nifH* PCR products was analyzed by electrophoresis in 2.0% (w/v) agarose gel and visualized via Bio-Rad UV transilluminator after staining gels with ethidium bromide. The initial sequencing of *nifH* PCR products was performed by Macrogen Laboratories (Seoul, South Korea). The sequences were then analysed using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) programme of the National Centre for Biotechnological Information (NCBI) to determine presence of the *nifH* gene.

Cloning and Analysis of Partial 16S rDNA Sequences

The partial sequences of 16S rDNA were amplified from the genomic DNA of the isolates using the forward primer UP2 5'-GGG CCC CCG YCA ATT CCT TTG ART TT-3' and the reverse primer URP 5'-GTG CCA GCM GCC GCG GTA A-3', as described by Bavykin *et al*. (2004). The PCR mixture consisting of 0.5 µl genomic DNA, 5 µl 10xPCR buffer, 3 µl 25mM $MgCl_2$, 2 µl 25pmol forward primer, 2 µl 25pmol reverse

primer, 1 µl 10mM dNTP, and 0.25 U Taq DNA polymerase was brought to a final volume of 50 µl with deionized distilled H_2O. The PCR profile conditions were as follows: an initial denaturation at 95°C for 5 min, 30 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 35 s, and final extension at 72°C for 10 min. The PCR products were separated by electrophoresis in 2.0% agarose gel. The 16S rDNA sequences obtained from PCR amplification were then ready for purification and cloning.

To accomplish this, the amplified PCR products of the partial 16S rDNA sequences were purified with Promega PCR Purification Kit (Madison, Wisconsin, USA) and cloned with *E. coli* JM109. The purified PCR products were ligated into pGEM-T Easy Vector Systems (Promega, USA) containing 1 µl 50ng μl^{-1} pGEM-T vector, 5 µl 2X rapid ligation buffer, 1 µl 3U μl^{-1} T4 DNA ligase, with a final volume of 10 µl and incubated overnight at 4°C. The ligation products were transformed into *E. coli* JM109 and cultured on Luria-Bertani agar media containing ampicillin (100 µg ml^{-1}), IPTG (0.1 M) and X-Gal (50 µg ml^{-1}). This was followed by achieving plasmid extraction from the white colonies using Wizard Plus SV Minipreps DNA Purification System (Promega, USA). The digestion of recombinant plasmid pGEM-T was verified using restriction enzymes *Not*1 and *EcoR*1 to confirm the ligation of partial 16S rDNA fragments before sequencing by Macrogen Laboratories. In order to identify each isolated strain, the closest genetic match was compared to those in the GenBank database in NCBI via nucleotide-nucleotide BLASTn programme.

RESULTS

Isolation and Identification of Microsymbionts

A total of thirteen microsymbionts were successfully isolated from the root nodules of *M. bracteata.* The isolates were grouped as Gram negative and positive strains. The results also showed that the isolated microsymbionts were 1.2-3.0 (length) x 0.5-0.9 (width) µm in size and varied in shape such as rod, short-rod, curved, straight and coccobacilli shapes, when observed under microscope. These isolates were differentiated by their growth rate into either fast-growing (3 days) or slow-growing (5 days) bacteria. The fast-growing bacteria (isolates USM-A11, USM-B9, USM-B12, USM-B20, USM-C4 and USM-C9) were observed as acid producers, while the slow growers (isolates USM-B4, USM-B14, USM-B15, USM-B19, USM-C1, USM-C2 and USM-C8) were alkaline producers, based on the changes of pH in YEMA incorporated with BTB. Most of the strains failed to absorb the red colour from RC.

Observation of N_2-fixation, Nodulation and Plant Growth

The isolates that showed positive symbiotic N_2-fixation activities based on H_2 evolution in the *M. bracteata* host plants were (in order of highest to lowest rate): USM-A11, USM-C1, USM-B19, USM-C2, USM-C9, USM-B14, USM-B20, USM-B15, USM-B4, USM-C4 and USM-B9; these and their

respective rates are presented in Fig.1. The N_2-fixation rates ranged from 24.1 to 78.5 μmol N_2 h^{-1} g^{-1} nodule dry weight. In this process, the nitrogenase enzyme catalyzes the reduction of N_2 into NH_3 and involves a successive allocation of electrons, together with evolution of H_2. For isolates USM-B12 and USM-C8, no fixation of N_2 was detected although both isolates could enhance plant growth. This non-detection was probably due to the presence of hydrogenase enzyme (Hup^+) uptake, which can recapture the H_2 evolved within the nodule.

Most of the isolates successfully developed diverse nodule shapes on *M. bracteata* roots, such as ovoid, cylindrical, lobed and irregular. The nodules were in various shades of black, dark-brown and reddish-brown. The isolated microsymbionts successfully infected the roots and nodulated the host plants, which then allowed the N_2-fixing process to supply the N source required for plant growth, as shown in the growth parameters listed in Table 1. Effective N_2-fixation activity by the microsymbionts (especially USM-B9, USM-B14, USM-B19, USM-B20 and USM-C2) could be suggested as the reason for the increases in leaf protein and chlorophyll content and in the biomass of the whole plants and nodules. The results derived for the leaf protein content indicated that the plants inoculated with isolates USM-B4 and USM-C2 recorded the highest protein content (57.91-59.88 mg BSA ml^{-1} protein) and had a significantly different effect compared with Control-2 (Table 1). Thus, the inoculation process with these potential isolates was important in fixing N_2 and in increasing the leaf protein content. In addition, the results for the leaf chlorophyll showed that the plants inoculated with isolates USM-B9, USM-B12, USM-B15, USM-B19 and USM-B20 produced higher leaf chlorophyll content as compared to Control 2. Similar results were also

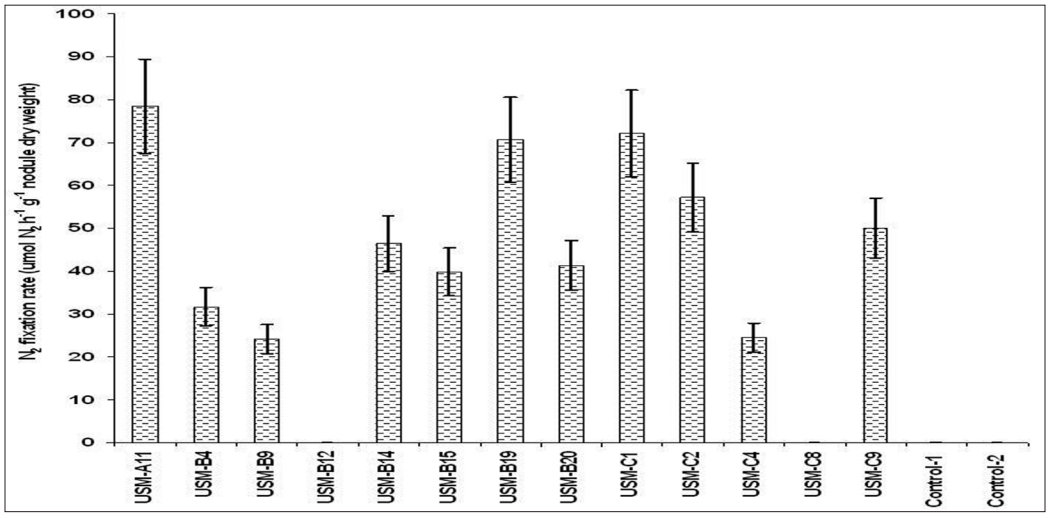

Fig.1: The influence of diazotrophic microsymbionts on the N_2 fixation rate of Mucuna bracteata at D_{60}

TABLE 1
Bacterial identification and influence of diazotrophic microsymbionts on the growth of *Mucuna bracteata* on the day of harvest (D_{65})

Identified Bacteria				Plant Growth Parameters			
						Dry Weight	
Class	Genus	Isolate/ Treatment	Similarity of identification (%)	Leaf Protein Content (mg BSA/ ml protein)	Leaf Chlorophyll Content (chlorophyll/ mg leaf fresh weight)	Whole Plant (g)*	Nodules (mg)*
Alphaproteobacteria							
	Brevundimonas sp.	USM-B4	100	57.91 b	0.21 a	1.84 ab	160.20 c
	Brevundimonas sp.	USM-C2	99	59.88 b	0.27 a	3.04 ab	85.85 abc
Betaproteobacteria							
	Achromobacter sp.	USM-B9	98	26.62 ab	1.08 c	3.95 b	142.50 bc
	Achromobacter sp.	USM-C8	100	41.24 ab	0.24 a	1.97 ab	46.20 ab
	Burkholderia sp.	USM-B15	100	17.92 ab	0.97 c	2.37 ab	93.00 abc
	Burkholderia sp.	USM-B20	99	27.98 ab	1.12 c	3.91 b	173.75 c
	Burkholderia sp.	USM-C9	100	18.63 ab	0.88 bc	1.68 ab	111.11 abc
Gammaproteobacteria							
	Stenotrophomonas sp.	USM-B14	99	17.38 ab	0.24 a	3.78 b	129.57 bc
Bacilli							
	Bacillus sp.	USM-A11	100	34.34 ab	0.17 a	2.72 ab	80.38 abc
	Bacillus sp.	USM-B12	99	40.97 ab	1.06 c	2.23 ab	168.75 c
	Bacillus sp.	USM-B19	99	22.76 ab	1.06 c	3.05 ab	127.25 bc
	Bacillus sp.	USM-C1	99	37.33 ab	0.25 a	1.31 ab	56.85 abc
	Bacillus sp.	USM-C4	99	28.30 ab	0.79 bc	2.91 ab	157.00 c
Control							
	+ N, - microsymbiont	Control-1		31.35 ab	1.10 c	2.44 ab	25.00 a
	- N, - microsymbiont	Control-2		6.87 a	0.54 ab	1.05 a	21.75 a

Note: Bacteria were identified by matching 16S rDNA sequences with those in BLASTn (Basic Local Alignment Search Tool) online database, National Center for Biotechnology Information (NCBI). Values for growth parameters are the means of four replications; for each growth parameter, values with the same letter(s) are not statistically significant at the Tukey probability of $p<0.05$. * Data were transformed to Log10 before being analyzed with SPSS V. 15.

recorded for the control plants i.e. Control-1 (+nitrogen, -inoculum), suggesting the ability of the inoculated microsymbionts in providing the host plant with fixed N_2 was equivalent to the plants receiving nitrogen fertilizer (Table 1).

Plants inoculated with USM-B9, USM-B14 and USM-B20 grew well and showed vigorous growth. Thus, totals of the plant dry weight were higher and showed a significant effect compared to the plants in Control 2 (Table 1). The experiment indicated that the plants inoculated with USM-B20 recorded the highest mean value for nodule dry weight at 173.75 mg plant^{-1}; a similar high nodule dry weight was also recorded for the plants inoculated with isolates USM-B4, USM-B12 and USM-C4 (Table 1). In addition, *M. bracteata* treated with isolate USM-B20 recorded the highest

mean values for the plant dry weight and chlorophyll content. Therefore, N_2 that was fixed by the microsymbionts in the root nodules provided an N source for *M. bracteata* that showed an increase in plant growth.

PCR Amplification and Sequencing of nifH Gene

Genomic DNA was successfully extracted from the isolated microsymbionts. The N_2-fixing ability of the potential microsymbionts was verified by the amplification of *nifH* fragments through PCR analysis. Since dinitrogenase reductase enzyme was encoded by the *nifH* gene, this gene was amplified and sequenced in representative isolates. The amplification of *nifH* region with degenerate primers yielded a single band of the expected size (approximately 380 bp) using primer *nifH*3, as suggested by Choo *et al*. (2003). The results indicated that the primer set *nifH*3 was suitable to amplify *nifH* fragments at 380 bp with a few optimized PCR protocols for isolates USM-A11, USM-B4, USM-B9, USM-B12, USM-B19, USM-C1, USM-C4 and USM-C8, respectively. Hence, the nifH fragment (~380 bp) was amplified in *Rhizobium leguminosarum* ATCC 10004 (a positive rhizobia strain) and identified as a nitrogenase iron protein (*nifH*) gene (90% similarity) using the BLASTn programme in NCBI (gene bank accession number FJ263754.1). The primer *nifH*3 was found to be unsuitable for amplifying the *nifH* fragments for the remaining isolates. Therefore, the *nifH* fragments for isolates USM-B14, USM-B15, USM-B20, USM-C2 and USM-C9 were amplified using primer *nifH*4 and exhibited *nifH* fragments at 550 bp, 650bp, 350 bp, 450 bp and 900 bp, respectively. The sequencing results confirmed that the *nifH* fragments of USM-B14 (93% similarity), USM-B20 (100% similarity) and USM-C2 (96% similarity) were nitrogenase iron protein (*nifH*) with the gene bank accession numbers AY787541.1, AJ010288.1 and GU433550.1, respectively, in the NCBI database. Nevertheless, the amplified *nifH* fragments from isolates USM-B15 and USM-C9 failed to show the presence of nitrogenase iron protein (*nifH*) based on the sequencing results.

Cloning and Sequencing of Partial 16S rDNA

Further analysis using partial 16s rDNA sequences was performed to recognize and confirm the identity of the isolated microsymbionts (Table 1). Identification via 16s rDNA sequence analysis is one of the most effective tools for identifying bacteria. In this study, the observation of the root nodules of *M. bracteata* revealed that they contained diazotrophic rhizobial and non-rhizobial microsymbionts. The partial 16S rDNA fragments was succesfully amplified at 450 bp. This fragment was successfully ligated into p-GEMT and cloned with *E. coli* JM109. Based on the BLASTn result in NCBI, the isolates USM-B15, USM-B20 and USM-C9 were identified as *Burkholderia* sp. with 100% similarity, the isolates USM-B4 and USM-C2 as *Brevundimonas* sp. with

99-100% similarity, the isolates USM-B9 and USM-C8 as *Achromobacter* sp. with 98-100% similarity, the isolate USM-B14 as *Stenotrophomonas* sp. with 99% similarity, and the isolates USM-A11, USM-B12, USM-B19, USM-C1 and USM-C4 as *Bacillus* sp. with 99-100% similarity (Table 1). Among the identified strains, several microsymbionts were determined as *Betaproteobacteria* (*Burkholderia* sp. and *Achromobacter* sp.). The identified diazotrophic microsymbionts obtained from *Alphaproteobacteria* was *Brevundimonas* sp. and this was *Stenotrophomonas* sp. from *Gammaproteobacteria*. These findings are similar to those in other reports, in which the bacteria outside the family of *Rhizobiaceae* and *Alphaproteobacteria* have been observed to produce nodules in legumes (Chen *et al*., 2005; Pandey *et al*., 2005; Elliott *et al.,* 2007b).

DISCUSSION

The recovered nodules from *M. bracteata* in this study were seen as being active in N_2-fixation. This was because their internal colouration was pink-red, showing that the root nodule bacteria were able to produce effective nodulation and N_2-fixation activity (Somasegaran & Hoben, 1985; Ojo, 2001). The microsymbionts showed diversity in classification and in response to the tests carried out. The isolates that produced blue colour on YEMA containing BTB were alkaline producers, while some isolates produced yellow colour that showed them to be acid producers. The changes of pH on YEMA were detected by incorporating BTB as a pH indicator in agar medium for the rhizobia. The fast growing isolates lowered the pH of the YEMA + BTB causing the agar to turn yellow within 3 days. In addition, these fast growing rhizobia had a mean generation time of 24 hours (Keyser *et al*., 1982; Anand & Dogra, 1991). In contrast, the slow growing isolates increased the pH and turned the media to blue within 5 days. Meanwhile, RC was added into YEMA to distinguish between the rhizobia and contaminants (Vincent, 1982). From this study, many of the isolates from *M. bracteata* failed to absorb the red colour from RC and this therefore indicated that the strains could be qualified as the rhizobial species. Additionally, most of the alkaline-tolerant strains were recognized from this identification. Consequently, further experiments could allow for the identification of these isolated microsymbionts in terms of their genetic identity and characteristics.

In the nodulation screening, all the 13 isolates were used to inoculate *M. bracteata.* This experiment was conducted under N-free conditions (except for the Control 1) as the main goal was to observe the ability of these microsymbionts to enhance the N_2-fixation activities in *M. bracteata.* The results of this study indicated that the nodulation and N_2-fixation activity increased plant growth parameters, such as plant biomass, protein and chlorophyll contents as compared to the Control-1 (+N, -microsymbiont) and Control-2 (-N, -microsymbiont). These microsymbionts successfully infected the roots and nodulated the host plants, thus allowing the N_2-fixation process to supply the N source required for plant growth**.**

This type of effective symbiotic relationship may explain why this legume has widely been used as a resource in the agricultural ecosystems. However, these benefits extend beyond the plant itself. The production of higher plant biomass and protein content is advantageous to the soil in terms of providing more decomposing organic matter, especially N nutrients, to immature crops in fields and plantations. Additionally, the vigorous growth of *M. bracteata* forms a thick leafy canopy close to the soil surface and consequently reduces soil temperature, leading to higher microbial activity and enrichment of the nutrient status of the soil (Zhao *et al.*, 1997; Mathews, 1998; Graham, 2008). Moreover, this type of symbiotic relationship provides a great compensation as it is not hazardous to the environment (Appunu & Dhar, 2008).

The N_2-fixing ability of the potential isolated microsymbionts was confirmed through the PCR analysis by amplifying the *nifH* fragment and sequencing in the representative strains. This gene is a key enzyme in N_2-fixation activity and is known as nitrogenase enzyme. In part of the nitrogenase enzyme region (nif gene), there is a *nifH* gene which is involved in encoding dinitrogenase reductase. Thus, this *nifH* PCR amplification and sequence analysis were undertaken to evaluate the diversity among the N_2-fixing microsymbionts in the root nodules of *M. bracteata*. The amplification of the *nifH* region with degenerate primers yielded a single band of the expected size using primers *nifH3* and *nifH,* as suggested by Choo *et al*. (2003). The results indicated that these primer sets were suitable to amplify the *nifH* fragments at several particular sizes. Thus, these molecular methods, based on the PCR detection of the *nifH* marker gene, have been successfully applied to describe the diazotroph populations in the nodules of *M. bracteata.*

The 16S rDNA sequence analysis is an effective tool to be used in identifying bacteria. The observation of the root nodules of *M. bracteata* revealed that they contained diazotrophic rhizobia and non-rhizobia based on the sequence analysis of partial 16S rDNA. From this experiment, several microsymbionts were identified as beta-class proteobacteria: *Burkholderia* sp. and *Achromobacter* sp. As for the alpha-class of proteobacteria, the identified bacterium was *Brevundimonas* sp.*,* while *Stenotrophomonas* sp. was from the gamma-class proteobacteria. Five isolated microsymbionts were identified as *Bacillus* sp. and this particular species is a non-rhizobial microsymbiont. This group of non-rhizobial microsymbionts was Gram-positive bacteria, as was detected in the Gram staining screening. These are Gram-positive and non-rhizobial strains but they may also be found co-existing with rhizobia in the root nodules of legumes.

In the recent years, the bacteria which do not belong to the Rhizobiaceae in *Alphaproteobacteria* were isolated. These bacteria are able to produce nodules and fix atmospheric N_2 (Willems, 2006; Chen *et al*., 2005; Sprent, 2008). These new nodulating bacteria have been identified

through 16S rDNA and are distinct from the Rhizobiaceae in the phylogenetic observation. For instance, *Brevundimonas, Devosia, Methylobacterium, Ochrobactrum* and *Phyllobacterium* from the *Alphaproteobacteria* class are also capable of nodulating and fixing N_2 in legumes.

The beta-class of the proteobacterial branch also contains nodulating bacteria such as *Burkholderia, Achromobacter, Cupriavidus* and *Ralstonia* (Willems, 2006). *Burkholderia* have recently been isolated from a variety of legumes (Chen *et al*., 2003a, 2005; Barrett & Parker, 2005, 2006; Elliott *et al*., 2007a, 2007b). The capabilities of these *Betaproteobacteria* in fixing N_2 and nodulating the host plant have been confirmed due to the existence of nodulation genes (*nod*) and *nif* genes which are similar to those of alpha-rhizobia and are located on a symbiotic plasmid (Chen *et al*., 2003b, 2005). From this experiment, *Burkholderia* spp. was found to be an effective inoculant for promoting plant growth. The isolates were able to fix N_2 and contained the *nifH* gene. *Burkholderia vietnamiensis* has previously been shown to be capable of enhancing plant growth, promoting indirect nodulation, serving as an antifungal, and aiding in phosphorus mobilization (Peix *et al*., 2001). Initially in the N_2-fixation studies, *B. vietnamiensis* was the only species identified as a N_2-fixing strain in the genus of *Burkholderia* and found to be associated with rice plants (Gillis *et al*., 1995). Apparently, a number of N_2-fixing *Burkholderia* species have recently been discovered in the natural environment and are associated with certain plants including legumes (Wong-Villarreal & Caballero-Mellado, 2010). *Burkholderia plantarii* was first identified by Azegami *et al*. (1987) and it is considered as plant pathogenic bacteria (Suarez-Moreno *et al*., 2008). However, several *B. plantarii* isolates have been used for rice seeding cultivation (Maeda *et al.,* 2006). Thus, the *Burkholderia* species have potential for agro-biotechnology applications. From this experiment, another beta-class proteobacterial isolate was obtained, i.e. *Achromobacter* sp. Benata *et al*. (2008) also successfully isolated *A. xylosoxidans* from the root nodules of *Prosopis juliflora* from the eastern area of Morocco. Moreover, their analysis of the *nodC* also yielded and revealed that the *Achromobacter* sp. isolates contained approximately 930 bp of the *nodC* gene based on the PCR amplification using appropriate oligonucleotide primers (Benata *et al.,* 2008). Thus, *Achromobacter* sp. has contributed to broadening the new legume-nodulating-bacteria taxonomy. *Stenotrophomonas* sp. [formerly known as *Xanthomonas* sp. (Juhnke & Des Jardin, 1989) and as *Pseudomonas* sp. (Swings *et al*., 1983)] has useful properties in the biological control of soil-borne plant disease, bacterial microflora in soil and in the plant rhizosphere (Lambert *et al*., 1987). Kan *et al*. (2007) isolated *S. maltophilia* from the root nodules of herbaceous legumes grown in Tibet, China, as well as *Rhizobium, Sinorhizobium, Mesorhizobium* and *Phyllobacterium.* Likewise, this strain is also associated with the roots of another

legume plant, *Astragalus bisulcatus* (Di Gregorio *et al*., 2005; Kan *et al*., 2007).

Results from partial 16S rDNA sequence analysis also confirmed that five *Bacillus* sp. microsymbionts were obtained from the root nodules of *M. bracteata.* This non-rhizobial species is considered as endophytic bacteria, which live within the plant tissues and are not harmful to the plant host (Kobayashi & Palumbo, 2000). Meanwhile, the presence of *B. thuringiensis* in legume plants has been shown to absorb nutrients from soil and inhibit soil-borne pathogens and insect pests (Chattopadhyay *et al.,* 2004; Kuklinsky-Sobral *et al*., 2004; Reyes-Ramirez *et al*., 2004; Taghavi *et al*., 2005; Wang *et al*., 2006; Pandey & Maheshwari, 2007), as well as increase overall plant growth (Andrews & Harris, 2000). The inoculation of *Phaseolus vulgaris* L. with a combination of *Bacillus* spp. and *Rhizobium* sp. was shown to promote root nodulation and other beneficial interactions (Karanja *et al*., 2007). Similarly, Bai *et al*. (2002) reported that *B. thuringiensis* could enhance root nodulation and plant growth in soybean when applied as a co-inoculum with *Bradyrhizobium japonicum.*

In addition, Mishra *et al*. (2009) showed that the co-existence of rhizobial and non-rhizobial plant-growth-promoting strains in leguminous plants might improve nodule production and N_2-fixation activity. In bacteria-legume symbioses, enhancement of N_2-fixation in the host plant is the most important factor. Thus, in this experiment, plants inoculated with *Bacillus* spp. (USM-A11, USM-B19 and USM-C1) showed better N_2-fixation, which resulted in increased plant growth, even when it was under N-free conditions. In addition, the *nifH* gene fragments were successfully amplified and studied from each *Bacillus* sp. isolate to aid in understanding the ability of these isolates in enhancing the growth of *M. bracteata*.

CONCLUSION

Various microsymbionts in the present study showed potential to enhance plant growth and N_2-fixation in *M. bracteata*. From partial 16S rDNA sequence analysis, the isolated strains of microsymbionts from the nodules of *M. bracteata* exhibited a species-rich variety. This suggests that the actual diversity of bacteria that can nodulate this legume is higher than expected, and this includes the bacteria outside the *Alphaproteobacteria* class. Such non-rhizobial bacteria deserve further study in terms of their species identify, long-term effects on plant growth, biochemical interactions with other endophytic bacteria, and potential for use in agro-biotechnology applications.

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Herpetofauna of Peta Area of Endau-Rompin National Park, Johor, Malaysia

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ABSTRACT

The amphibians and reptiles of Peta, Endau-Rompin, Johor, Malaysia were briefly investigated during a scientific expedition organized by the School of Biological Sciences, Universiti Sains Malaysia from 17 to 23 August 2008. A total number of 47 species of amphibians and reptiles were recorded during the survey. Out of this number, 25 species of amphibians from 15 genera and 6 families were found. Meanwhile, six species of frogs are considered as commensal species and could easily be found in disturbed areas, and the others are forest frogs. A single species of caecilian, namely, *Caudacaecilia nigroflava,* from the family Ichthyophiidae was also recorded. As for the reptiles, 11 species of snakes from three families and 11 species of lizards from four families were recorded to inhabit the area. This report constitutes the first checklist of herpetofauna of Peta, Endau-Rompin, Johor, covering 24.3% of 103 frogs, 14.1% of 78 snakes and 10.2% of 108 lizard species that have been reported in Peninsular Malaysia thus far.

Keywords: Peta, Endau-Rompin, Johor, Peninsular Malaysia, amphibian, reptilian

INTRODUCTION

Endau-Rompin National Park (approximately 49,000 ha) is located in the southern part of Peninsular Malaysia and has been gazetted as a National Park since 1993. This is the second National Park established in Peninsular Malaysia with the main purpose of preserving the natural heritage of the country. The Endau-Rompin National Park area includes the southern part of the state of Pahang and also the northern part of the state of Johor and it is managed by Johor National Park Corporation. Gunung Besar (1036 m a.s.l.) is the highest peak

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and located in the western part of the park. The main river, i.e. Sungai Endau and its tributaries drain the area and empty into the South China Sea near the small town of Endau. This million year old tropical rain forest in Endau-Rompin provides various types of microhabitats that act as sanctuaries for the wildlife which includes amphibians and reptiles.

The park is a home for at least 95 species of mammals, 250 species of birds, and 76 species of fish (Chew, 2007). Several endangered species, such as *Dicerorhinus sumatrensis* (Sumatran Rhinoceros), *Elephas maximus* (Asian Elephant), *Panthera tigris* (Malayan Tiger) and *Tapirus indicus* (Malayan Tapir), were also found here. Other species of mammals, birds and fishes, such as *Sus barbatus* (Bearded pigs), *Felis bengalensis* (Leopard Cat), *Hystrix brachyuran* (Common Porcupine), *Argusianus argus* (Great Argus Pheasant), *Buceros Rhinoceros* (Rhinoceros Hornbill), *Wallago leerii* (Tapah fish), *Tor tambroides* (Kelah fish) and *Scleropages formosus* (Green Arowana fish), were also found to inhabit the forests and the rivers.

Previous studies on herpetofauna in Peninsular Malaysia by several scientists at different locations have shown various numbers of amphibians and reptiles. For example, 54 species of amphibians and reptiles were found in Ulu Endau (Kiew, 1987), 33 species of amphibians and 34 species of reptiles in the western region of Endau-Rompin (Daicus & Hashim, 2004), 24 species of amphibians and 51 species of reptiles in Temenggor (Kiew *et al*., 1995), 19 species of amphibians and 41 species of reptiles in Tasek Bera (Norsham *et al.,* 2000a), 9 species of amphibians and 17 species of reptiles in North Belum forest (Norsham *et al.,* 2000b), 13 species of amphibians in Wang Kelian (Ibrahim *et al*., 2001), 24 species of amphibians and 88 species of reptiles in Seribuat Archipelago (Grismer *et al.*, 2006b), as well as 16 species of amphibians and 23 species of reptiles in Langkawi (Ibrahim *et al*., 2006). Various factors such as duration of sampling period, area of coverage, sampling technique, topography, weather, types of microhabitat and activity pattern have been reported to influence the number of species recorded in each area (Inger, 2003).

Similarly, some continuous studies have also shown increased numbers of the amphibian and reptile species in Peninsular Malaysia. An early record by Berry (1975) showed that there were 83 species of amphibians inhabiting Peninsular Malaysia; however, within 30 years, this number has increased to 100 species (Inger, 2005) and 103 species of amphibians (Norhayati, 2009). The increase in the number of amphibian and reptile species clearly shows that Malaysian forests are very rich in herpetofaunal assemblage. A number of frogs have recently been described; these include *Leptolalax kajangensis* (Grismer *et al.,* 2004b), *Odorrana monjerai* (Matsui & Ibrahim, 2006), *Ansonia endauensis* (Grismer, 2006), *Ansonia latiffi* and *Ansonia jeetsukumarani* (Wood *et al*., 2008), *Gastrophrynoides immaculatus* (Chan *et al*., 2009) and *Leptolalax kecil* (Matsui *et*

al., 2009). As for reptiles, many new species have also been discovered recently from the forests (Leong & Grismer, 2004; Das & Grismer, 2003; Grismer, 2005, 2008a; Grismer & Das, 2006; Grismer *et al*., 2006a, 2008a, b; Grismer & Chan, 2008; Grismer & Norhayati, 2008). Despite the recent discoveries, many areas in Peninsular Malaysia have not been canvassed for their herpetofauna. Therefore, the main objective of this study was to search for and record the amphibian and reptile species that inhabit Peta, Endau-Rompin, as well as to build a baseline data for this particular vertebrate group.

MATERIALS AND METHODS

A herpetological survey of Peta, Endau-Rompin, Johor, Malaysia (Fig.1 and Fig.2) was carried out during a six-day Scientific Expedition, organized by the School of Biological Science, Universiti Sains Malaysia (USM), starting from 17 to 23 August 2008. Kampung Peta, (2^0 54'N, 103^0 41'E, < 300 m a.s.l.) is located in the eastern region of the Endau-Rompin National Park and it is around 52, 106 and 726 km from Kahang, Kluang and USM (Penang), respectively. From Kahang town, it can be reached by four-wheel drive vehicles, which will have to pass through oil palm estates and a primary rain forest before reaching the destination. The lowland dipterocarp forest is dominated by *Dipterocarpus sublamellatus* (Keruing Kerut), *Shorea leprosula* (Meranti Tembaga), *Shorea ovalis* (Meranti Kepong), *Shorea curtisii* (Meranti Seraya), *Neobalanocarpus hemii* (Cengal) *Dryobalanops aromatica* (Kapur), *Koompassia excelsa* (Tualang) and *Alstonia angustiloba* (Pulai). Within the forest, there is a great diversity of other plants species, such as palms, climbers, epiphytes, bamboo, herbs, ferns and fungi.

The collection of amphibians and reptiles was done around the Nature Education Research Centre (NERC) (2^0 53'N, 103^0 41'E), Kuala Jasin (2^0 53'N, 103^0 37'E) and Anak Jasin River (2^0 52'N, 103^0 36'E). All the specimens were collected during day and night (20:00-23:00 hours) along the forest trails, forest floor, swamps, streams, rivers, ponds and puddles. A sampling team comprising of four persons searched and collected the specimens by hand or sweep nets. For the night sampling, torch lights and head lamps were used to locate the specimens. For identification purposes, references such as those by Berry (1975), Denzer and Manthey (1991), Inger and Stuebing (1997), Cox *et al.* (1998), Stuebing and Inger (1999), Frost (2010) and Haas *et al*. (2010) were used. All the captured specimens were fixed with 10% formalin and preserved in 70% ethanol and later deposited at the School of Pharmaceutical Sciences (USM) for future references. The measurements of snout-vent length (SVL) for the frogs and total length (ToL) for the lizards were done using a Vernier caliper, whereas the measurement of the snakes (ToL) was only done by estimation because no specimen was captured, except for *Dendrelaphis pictus.*

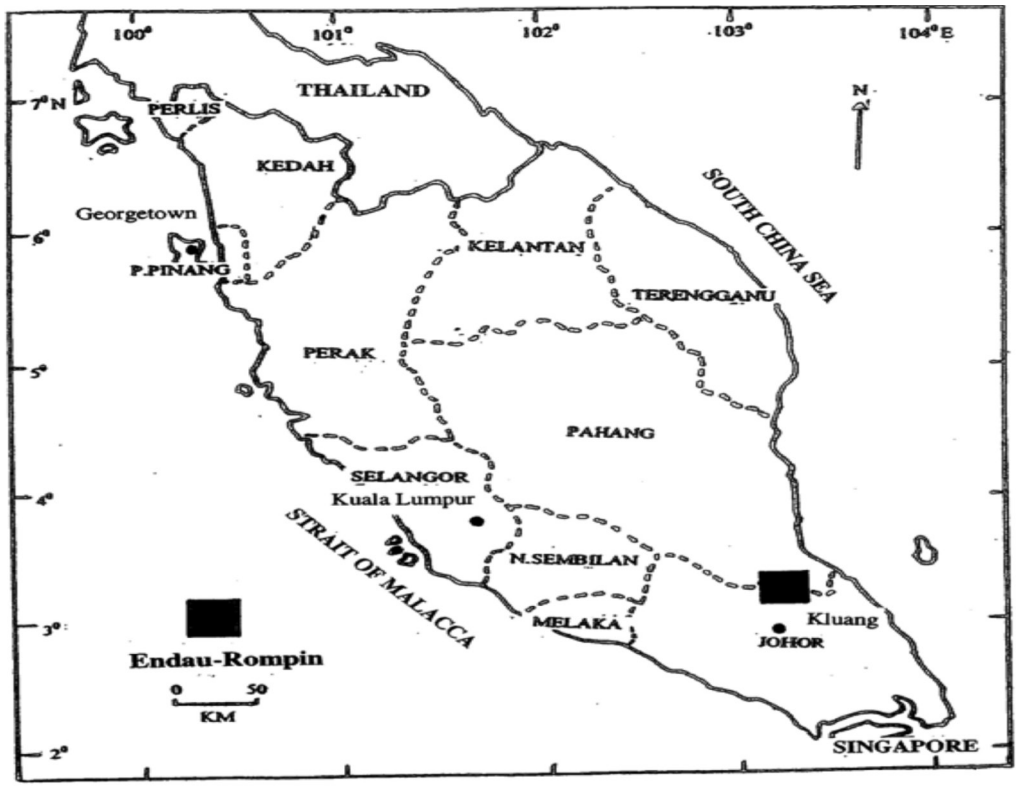

Fig.1: Location of Taman Negara Endau-Rompin

Nature Education Research Centre (NERC)

NERC is located 3 km from Kampung Peta and can be reached by road or river. The research centre is about 1 km X 1 km and is the main centre for the scientists to do their research activities. The facilities provided at the centre include chalets, dorminatories, a multi-purpose hall, a dining hall, an office building, a laboratory, a library and a boat jetty. Around the area, there are artificial ponds, swamps, small streams, bushes and open areas, and it is also surrounded by lowland dipterocarp forest. The sampling activities were done at night and during the day, as stated above.

Kuala Jasin

Kuala Jasin is located some 9 km from NERC and it can be reached by road and river. This is the main recreational area with chalets and camping site facilities for the tourists. In Kuala Jasin, the sampling was done along the Endau River (300 m) and around 3 to 4 m away from the river bank. The sampling was also carried out around the swampy areas, freshwater marsh and along the Janing Barat trail up

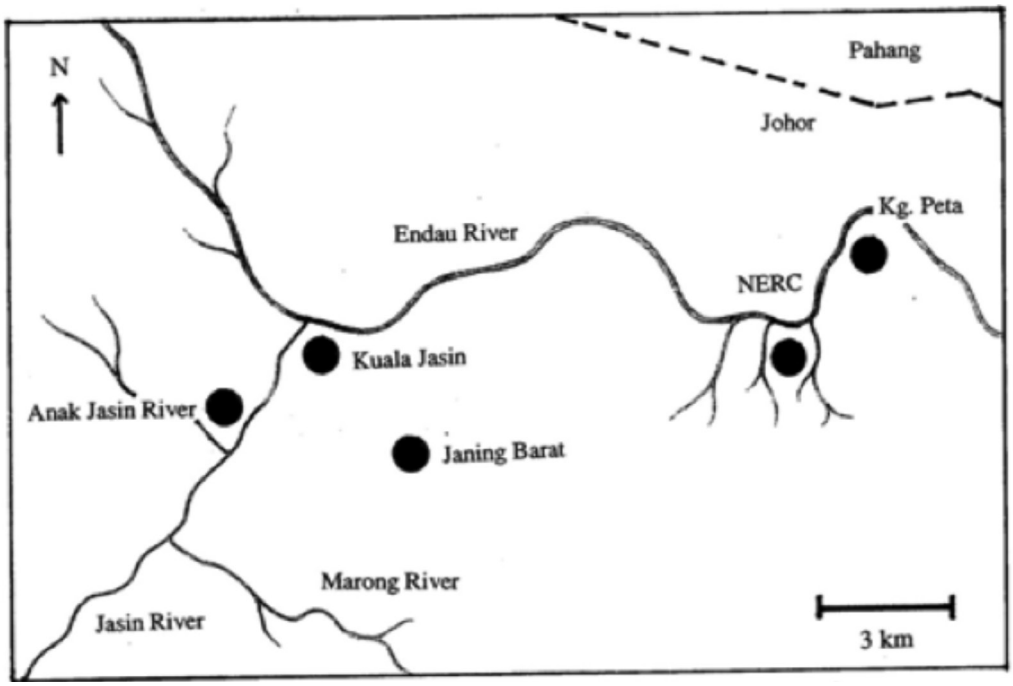

Fig.2: Location of NERC, Kuala Jasin and Anak Jasin River

to the top (450 m a.s.l.). This area is also surrounded by a lowland dipterocarp forest with abundant of palm trees.

Anak Jasin River

Anak Jasin River is about 3 km from Kuala Jasin and can be reached by walking through the Kuala Marong trail. The river flows into Jasin River, Endau River and empties into South China Sea. The sampling was carried out along 300 m of Anak Jasin River and around 3 - 4 m away from the river bank, apart from around the puddles, rain pools and small streams near the river.

RESULTS

Twenty five species of amphibians, 11 species of snakes and 11 species of lizards were found and collected during the six-day expedition. The species and a brief description of their habitat are summarized in Table 1. Meanwhile, the number of the species observed, captured, sex, age, and sizes of the amphibians and reptiles are presented in Table 2.

DISCUSSION

The million year old tropical rain forest in Endau-Rompin provides a variety of environments such as rivers, streams, waterfalls, swamps, freshwater marsh, forest floor and tree canopy that are apparently suitable for the organisms to live in and breed, including the amphibians and reptiles. The six-day expedition revealed 25 species of amphibians and 22 species of

TABLE 1
Amphibians and Reptiles Checklist of Peta and Western Region of Endau-Rompin, Johor

In Peta Area of Endau Rompin (Present Study)		In Western Region of Endau Rompin (Daicus & Hashim, 2004)	
Taxa		**Habitat**	
AMPHIBIA			
Bufonidae			
Ansonia leptopus	-		+
Duttaphrynus melanostictus	-		+
Ingerophrynus parvus	+	Swampy areas, river bank	+
Phrynoidis aspera	+	Perch on the rocks in the river, swampy areas	+
Pedostibes hosii	+	Perch on tree branches near small stream	-
Pelophryne breviceps	-		+
Pelophryne signata	-		+
Dicroglossidae			
Fejervarya cancrivora	-		+
Fejervarya limnocharis	+	On the grass, near pond	+
Limnonectes blythii	+	Swampy areas, river bank	+
Limnonectes kuhlii	+	Perch on the rocks, small rocky stream	-
Limnonectes laticeps	+	Perch on the rocks, small rocky stream	+
Limnonectes malesianus	-		+
Occidozyga laevis	+	Swampy areas, rainpools, puddles	+
Occidozyga lima	+	Swampy areas, rainpools, puddles	-
Ranidae			
Amolops larutensis	+	Rock crevice in cascade area, waterfall	+
Hylarana erythraea	+	Near pond	-
Hylarana glandulosa	+	Swampy areas, river bank	+
Hylarana labialis	+	Perch on tree branches adjacent to the river, on rocks, swampy areas	+
Hylarana laterimaculata	+	Under decaying wood, swampy areas	-
Hylarana picturata	+	Freshwater marshes, small streams in the forest	+
Odorrana hosii	+	Perch on big rocks in fast flowing streams	+
Megophryidae			
Leptobrachium hendricksoni	+	Swampy areas, forest trails, under dead leaves	-
Megophrys nasuta	+	Small streams in the forest	+
Microhylidae			
Chaperina fusca	-		+
Kalophrynus palmatissimus	-		+

Table 1 (*continued*)

Kalophrynus pleurostigma	-		+
Kaloula baleata	-		+
Kaloula pulchra	+	In cement drain	+
Metaphrynella pollicaris	-		+
Microhyla annectens	-		+
Microhyla butleri	+	Tall grass and shrubs	-
Microhyla heymonsi	+	On the road, roadside	+
Microhyla berdmorei	+	Under dead leaves, forest trails	+
Microhyla ornata	-		+
Rhacophoridae			
Polypedates leucomystax	+	Pond and shrubs, in the toilet	+
Polypedates macrotis	+	Perch on tree branches near the road	+
Rhacophorus nigropalmatus	-		+
Rhacophorus pardalis	-		+
Ichthyophiidae			
Caudacaecilia nigroflava	+	Ephemeral pond with a lot of dead leaves	+
REPTILIA			
Snake			
Elapidae			
Bungarus flaviceps	+	Near river bank	+
Naja kaouthia	+	Road kill	-
Colubridae			
Ahaetulla mycterizans	+	On tree branches, dense bush	-
Ahaetulla prasina	-		+
Boiga dendrophila	+	Swampy areas	-
Chrysopelea ornata	+	On tree trunk	
Chrysopelea paradisii	+	On tree branches	-
Dendrelaphis formosus	-		+
Dendrelaphis kopsteini	+	Forest trails	-
Dendrelaphis pictus	+	Edge of the pond	-
Macropisthodon rhodomelas	+	Forest trails	+
Rhabdophis chrysargus	-		+
Pythonidae			
Python reticulatus	-		+

Table 1 (*continued*)

Typhlopidae			
Typhlops diardi	-		+
Viperidae			
Trimeresurus wiroti	+	Near big tree buttress	-
Tropidolaemus wagleri	+	On tree branches along the forest trails	+
Lizard			
Agamidae			
Aphaniotis fusca	-		+
Bronchocela cristatella	+	On tree branches near the road	+
Calotes versicolor	+	Bush and dense shrubs	+
Draco blanfordii	-		+
Draco fimbricatus	-		+
Draco melanopogon	-		+
Draco obscurus	-		+
Draco volans	-		+
Gonocephalus bellii	-		+
Gonocephalus grandis	+	On tree trunk and leaves near fast flowing stream	+
Gonocephalus liogaster	-		+
Gekkonidae			
Cyrtodactylus consobrinus	+	Tree trunk, tree buttress	+
Cyrtodactylus pulchellus	-		+
Cyrtodactylus quadrivirgatus	-		+
Cyrtodactylus sworderi	-		+
Gekko gecko	-		+
Gekko smithii	+	On ceilings, walls and toilets	+
Gekko monarchus	+	On ceilings and walls	+
Gehyra mutilata	+	Dining hall, chalets	-
Hemidactylus frenatus	+	Dorm, dining hall, kitchen, toilets, chalets	-
Scincidae			
Eutropis longicaudata	-		+
Eutropis macularia	-		+
Eutropis multifasciata	+	Cement ditch, forest trails	+
Eutropis rugifera	-		+
Sphenomorphus scotophilus	+	Small stream in forest, dead stump	-
Varanidae			
Varanus nebulosus	-		+
Varanus rudicollis	-		+
Varanus salvator	+	Swampy areas, river banks	+

Table 1 (*continued*)

Freshwater turtle		
Bataguridae		
Heosemys grandis	-	+

Note:
+ Present
- Absent

TABLE 2
Number of observed, captured, sex, age and size of amphibian and reptile specimens of Peta, Endau-Rompin, Johor

Taxa	No. Obs.	No. Cap.	Sex	Age	Size
AMPHIBIA					
Bufonidae					
Ingerophrynus parvus	41	5	3 Male 2 Female	Adult	45-58 mm
Phrynoidis aspera	7	2	Unknown	Adult	134-152 mm
Pedostibes hosii	4	1	1 Male	Adult	92 mm
Dicroglossidae					
Fejervarya limnocharis	9	2	1 Male 1 Female	Adult	54-73 mm
Limnonectes blythii	3	1	Unknown	Adult	115 mm
Limnonectes kuhlii	3	3	Unknown	Adult	49-54 mm
Limnonectes laticeps	5	4	Unknown	Adult	42-48 mm
Occidozyga laevis	8	4	Unknown	Adult	27-34 mm
Occidozyga lima	2	1	Unknown	Adult	25 mm
Ranidae					
Amolops larutensis	25	6	Unknown	4 Adult 2 Juvenile	27-59 mm
Hylarana erythraea	7	2	1 Male 1 Female	Adult	65-92 mm
Hylarana glandulosa	15	1	Unknown	Adult	89 mm
Hylarana labialis	35	4	4 Male	Adult	55-68 mm
Hylarana laterimaculata	2	2	Unknown	Adult	48-57 mm
Hylarana picturata	5	1	Unknown	Adult	54 mm
Odorrana hosii	6	2	Unknown	Adult	108-121 mm
Megophryidae					
Leptobrachium hendricksoni	8	5	Unknown	Adult	42-64 mm
Megophrys nasuta	4	-	-	-	-

Table 2 (*continued*)

Microhylidae					
Kaloula pulchra	5	1	1 Male	Adult	82 mm
Microhyla butleri	8	3	Unknown	Adult	27-35 mm
Microhyla heymonsi	6	4	Unknown	Adult	24-37 mm
Microhyla berdmorei	1	1	Unknown	Adult	47 mm
Rhacophoridae					
Polypedates leucomystax	10	3	2 Male	Adult	55-64 mm
			1 Female	Adult	89 mm
Polypedates macrotis	1	1	Unknown	Adult	108 mm
Ichthyophiidae					
Caudacaecilia nigroflava	1	1	Unknown	Juvenile	184 mm
REPTILIA					
Snake					
Elapidae					
Bungarus flaviceps	1	-	Unknown	Adult	app. 800 mm
Naja kaouthia	1	-	Unknown	Adult	app. 750 mm
Viperidae					
Trimeresurus wiroti	1	-	Unknown	Juvenile	app. 280 mm
Tropidolaemus wagleri	1	-	Unknown	Juvenile	app. 320 mm
Colubridae					
Ahaetulla mycterizans	2	-	Unknown	1 Adult	app. 610 mm
				1 Juvenile	app. 350 mm
Boiga dendrophila	1	-	Unknown	Adult	app. 1520 mm
Chrysopelea ornata	1	-	Unknown	Adult	app. 670 mm
Chrysopelea paradisii	1	-	Unknown	Adult	app. 550 mm
Dendrelaphis kopsteini	1	-	Unknown	Adult	app. 980 mm
Dendrelaphis pictus	2	1	Unknown	Juvenile	app. 540 mm
Macropisthodon rhodomelas	1	-	Unknown	Juvenile	app. 410 mm
Lizard					
Gekkonidae					
Cyrtodactylus consobrinus	2	1	Unknown	Adult	215 mm
Gekko smithii	7	1	Unknown	Adult	296 mm
Gekko monarchus	6	2	Unknown	Adult	184-193 mm
Gehyra mutilata	5	1	Unknown	Adult	132 mm
Hemidactylus frenatus	35	2	Unknown	Adult	114-135 mm

Table 2 (*continued*)

Agamidae					
Bronchocela cristatella	1	-	Unknown	Adult	-
Calotes versicolor	4	1	Unknown	Adult	207 mm
Gonocephalus grandis	3	1	Unknown	Adult	268 mm
Scincidae					
Eutropis multifasciata	5	1	Unknown	Adult	174 mm
Sphenomorphus scotophilus	2	2	Unknown	Adult	133 mm
Varanidae					
Varanus salvator	6	-	Unknown	Adult	-

Note:
No. Obs. = Number observed
No. Cap. = Number captured

reptiles. For the amphibians, the number constituted 24.3% of the 103 amphibian (Norhayati, 2009) species reported in Peninsular Malaysia. As for the snakes and lizards, these covered 14.1% of 78 snake (Norhayati, 2009) and 10.2% of 108 lizard (Grismer, 2008b) species inhabiting Peninsular Malaysia.

Daicus and Hashim (2004) found 32 species of frogs, one species of caecilian, 25 species of lizards, eight species of snakes, and one species of freshwater turtles in the western region of Endau-Rompin. In this study (Peta area), 24 species of frogs, one species of caecilian, 11 species of lizards and 11 species of snakes were recorded. In particular, 15 species of amphibians were found in the western region, but not in the Peta area. Similarly, the seven species found in the Peta area were not found in the western region. Nonetheless, 18 species were found to be common in both places. The seven species of frogs found in the Peta area but not in the western region were *P. hosii, L. kuhlii, O. lima, H. erythraea, H. laterimaculata, L. hendricksoni* and *M. butleri.* As for the reptiles, 23 species recorded in the western region were not discovered in the Peta area. Likewise, 11 species recorded in Peta, were not found in Western Region, and 11 species were recorded in both the places. The 11 species of reptiles inhabiting Peta but not the western region were *N. kaouthia, T. wiroti, A. mycterizans, B. dendrophila, C. ornata, C. paradisii, D. kopsteini, D. pictus, G. mutilata, H. frenatus* and *S. scotophilus*.

The numbers of species recorded in Peta, Endau-Rompin are lower than those reported by Daicus and Hashim (2004) because of several reasons, especially the short duration of the survey period. The six-day exploration is apparently not enough to cover the entire forests, swamps, rivers and waterfalls in Peta. In particular, the present study covered only small areas around NERC, Kuala Jasin and Anak Jasin River. Other unvisited areas around Peta, especially areas deep in the forest, such as Tasik Air Biru, Upeh Guling and Buaya

Sangkut waterfalls, are suggested to be intensively explored so as to discover more species of amphibians and reptiles. For comparison purposes, Daicus and Hashim (2004) conducted a longer survey period (about 20 days) and covered more pristine areas such as Lubok Tapah, Lubok Merekek, Takah Tinggi Waterfall, Sungai Selor and Gunung Tiong.

Most of the frogs captured were of the riparian species due to the fact that the sampling areas were more focused to rivers, streams and swamps. Only two rhacophorids were found, namely *P. leucomystax* and *P. macrotis*, because of their arboreal characteristics. Others were not found as tree frogs, such as *R. nigropalmatus* and *R. pardalis*, live and forage high in the tree canopy and only come down to the forest floor during the breeding season. These two species usually choose wildlife (pig or rhinocerous) wallows in the forest floor as their breeding site (Inger & Stuebing, 1997). The arboreal toad, *Pedostibes hosii*, was found croaking from tree branches near a small river after heavy rain. These toads spend most of their time deep in the forest and only go to the river or pond for breeding. Two puddle frogs, i.e. *O. laevis* and *O. lima*, were found in the swamps and puddles near NERC and these frogs use this type of water bodies as their breeding sites.

From the total number of the amphibians, six species (including *F. limnocharis, H. erythraea, K. pulchra, M. butleri, M. heymonsi* and *P. leucomystax*) are considered as commensal species associated with human activities. These frogs have a generalized habitat and are commonly found in disturbed areas up to the forest edge. It is important to note that these species could be used as a bio-indicator to determine forest disturbances. Meanwhile, certain species such as *P. aspera, H. glandulosa* and *L. blythii* could adapt and are found in moderately disturbed forests. The others are typical forest frogs that have a specialist habitat and can be found only in the forest environment. The study was more focused on the frog fauna around the natural water bodies, such as streams and swamps, compared to forest floor and tree canopy. As a result, more riparian species were captured compared to the others. Thus, other methods of collection (e.g. pit-fall traps) are suggested to capture more forest floor fauna in future studies.

Several species of lizards, such as *G. mutilata, H. frenatus, C. versicolor* and *E. multifasciata*, could be easily found around NERC (base camp). These commensal species have a general habitat and can survive around human habitation. Among other, *Gehyra mutilata* and *H. frenatus* inhabit the buildings and chalets, while *C. versicolor* and *E. multifasciata* were found in the bushes, garden and open areas around NERC.

The others are forest lizards and their main habitat is in the forest. Sometimes, some species of lizards, such as the giant forest lizard, *G. smithii* and spotted lizards, *G. monarchus*, were found entering the buildings in NERC. They are usually found crawling on the wall and ceiling of the

building looking for insects at night. The availability of food might be the reason for this particular species to enter the buildings from the nearby forest. Meanwhile, species like *C. consobrinus* could be found perching on tree trunk, buttress or holes of tree stumps at night in the forest that are close to the rivers.

Agamid lizards, such as *G. grandis and B. cristata,* can be found perching on tree trunks and three branches near the stream in the forest. These species prefer primary and secondary forests but they can be found near the base camp at times. Meanwhile, species like *S. scotophilus* is active at day time and can be sighted foraging near the small stream in the forest. The water monitor, *V. Salvatore,* is also active at day time and can be found in almost all types of environment, especially near the swamps and rivers.

On the contrary, snakes were rather difficult to locate because of their elusive behaviour and camouflage characteristics. In this study, only 11 species of snakes were observed and most of them were sighted in the forest, specifically near the streams and swampy areas. Only four species of snakes, namely *A. mycterizans, D. pictus, T. wiroti* and *N. kaouthia,* were found around the base camp and forest edge. The current checklist of amphibians and reptiles in Peta area is by no mean complete, as more studies are definitely needed for that purpose.

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Rural Poultry Keeping in South Gezira, Sudan

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ABSTRACT

A study on rural poultry production, management and health was conducted at six randomly selected villages in the south district of Gezira state in central Sudan. Hundred rural farmers were interviewed using a set of questionnaire. A scavenging system is commonly practiced by the farmers in all villages. Females contributed significantly the highest percentage of the farmers, with 64% versus 46% (males). The farmers prefer local breeds (77% of farmers). The majority of the farmers who rare local breeds are illiterate or with merely primary education (43/77), and they also do not use proper housing or feeding the chickens, vaccination against diseases, and with no use of medication and are not willing to vaccinate. Moreover, they also do not provide water, and even if they do, it is usually dirty as they do not clean it. Meanwhile, the farmers who keep cross breeds are mainly secondary school or university graduates (13/23). This particular group provide a better managerial aspect in constructing a poultry house that provides poultry rations or household withdrawal plus grains or poultry ration. In addition, they are also vaccinated against Newcastle disease, use medication against external and internal parasites, provide feeders and drinkers and clean them periodically. The highest flock size (more than 70 chicken including young chicks) was found to be owned by more literate farmers who keep cross breeds as compared to the local breed kept by illiterate farmers (13/23 and 3/23 cross breeds were kept by more literate and illiterate farmers, respectively). The farmers keep local breeds mainly for self sustain (eggs and meat) and others keep cross breeds for income and mainly egg production. Hatchability percentage is slightly high in local breeds compared to cross breeds and is preferred during winter.

Keywords: Chickens, local breeds, farmers, scavenging, questionnaire, vaccination

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INTRODUCTION

Poultry keeping in the rural areas of Sudan is one of the most ancient household activities which are practised in both transhumant and in settled life areas. A family usually keeps a variable number of birds, from local breeds, around the homestead and no distinct system of poultry management is followed. The birds are kept free around the house compound and use the same shelter as that utilized by the family. The importance of village poultry keeping in the Sudan, as a factor contributing to the nutritional level of the family, is fully realised. Therefore, efforts are being made to promote poultry production under village conditions and to control diseases. These efforts were started by the establishment of demonstration units at provincial veterinary headquarters, educational centres and at agricultural pumping schemes. Then, a model poultry farm was established in Khartoum North, with the objective of providing good quality hatching eggs, graded cockerels and extension services to village poultry keepers. An advisory programme was also implemented to deal with the breeding, housing, feeding and management aspects of poultry production. Despite the government's efforts, no improvement has been made in the rural poultry production and the official attention has attracted commercial intensive poultry production and research work for improving the production of local breeds under an intensive system. This article reviews the information available in Sudan on the performance of the local breeds under intensive and traditional husbandry systems.

In nearly all African countries, poultry production in the rural areas is predominantly based on a free-range system utilising indigenous types of domestic fowl (Kitalyi, 1998; Host, 1988). The system is characterised by a family ownership of the birds. The birds are then left to scavenge in order to meet their nutritional needs. The feed resources vary depending on the local conditions and the farming system. Housing may not be provided (Huchzermeyer, 1973; Kuit *et al*., 1986; Atunbi & Sonaiya, 1994) and even if it is provided, local materials are usually used (Atunbi & Sonaiya, 1994). Management is very minimal with some variations of gender roles in the activities (Olayiwole, 1984; Achiempong, 1992). The health of the birds is not guaranteed because there are no disease control programmes. The birds are exposed to many disease conditions. Among other, the Newcastle disease has been noted as the most prevalent and devastating poultry disease in many African countries (Chrysostome *et al*., 1995). Parasites are also prevalent due to favourable conditions (Permin & Hansen, 1998). It was concluded that the major constraints affecting the rural poultry production are Newcastle disease and parasites, inadequate housing and poor feed supplementation, especially in the dry season (Illang *et al*., 2000). Women have important responsibilities in the rural poultry production in the two zones. A research work targeting at studying the rural poultry production in six villages in South

Gezira District was carried out with the overall objective of developing integrated and appropriate management and health interventions.

MATERIALS AND METHODS

Study Design

Six villages located in South Gezira District, Gezira State were randomly selected for this study. The total number of the farmers was 100, and these ranged from 14-20 per village.

Study Population

The study population included all the village chicken reared at the villages. The target groups were the local or indigenous fowl and the hybrids of exotic breeds and the local ones.

Data Collection

Questionnaire Survey

Information related to chicken management was obtained by interviewing the farmers or stakeholders in their homes, using a structured pre-tested questionnaire. The information included more than 32 parameters. The most important of which were the gender of the stakeholder, education level, flock type or breed, and flock size (hens, cocks, pullets and chicks). The managerial aspects included the housing system, as well as the uses of proper feeders and drinkers and cleaning them. The feeding system of chickens was also considered, while care and feeding of hens sitting on hatching eggs. The selection of hatching eggs, the best season of hatching, the days the hen sits on eggs and the chicks brooding time. The health questions involved the vaccination and medication against diseases and the farmers' willingness to vaccinate. The questionnaire also included the socioeconomic aspect in the purpose of chicken keeping, the most preferred product, the laying interval or the number of clutches, as well as the number of eggs per clutch and marketing availability.

Remarks

The farmers were given the opportunities to tell their problems and give any suggestions.

Data analysis

The data obtained were managed, collated, and analysed using SPSS Version-15 statistical software (SPSS Inc. Chicago). Meanwhile, a descriptive analysis was used to describe the sampled population in the study. The differences between the proportions were tested using the Chi square ($\chi 2$) analysis at the significance level of $\alpha = 0.05$. In addition, a cross tabulation concentrating on the level of education versus all the managerial aspects and health was also done in the study.

RESULTS AND DISCUSSION

As shown in Table 1, the females represented the highly percentage of poultry keepers in South Gezira district (77%). These are in agreement with of that of Illang *et al*. (2000). Nonetheless, no significant differences ($P>0.05$) were observed in the level of

TABLE 1
The effects of farmers' education level on the different managerial aspects of poultry keeping in South Gezira

Parameters	Level of Education					Total	Level of Significance
	Illiterate	Primary	Intermediate	Secondary	University		
Sex of Interviewer							
Male	7	11	5	6	7	36	0.722
Female	16	16	8	16	8	64	
The flock type and breeds							
Cross Breeds	3	6	1	6	7	23	0.092
Local Breeds	20	21	12	16	8	77	
The Total Flock Size							
Less than 30	5	3	1	2	0	11	
31- 50	11	5	4	3	0	23	
51- 70	4	6	0	5	2	17	0.003
More than 70	3	13	13	12	13	49	
System of Housing							
No access to housing	15	9	5	4	1	32	
Backyard small poultry pen	8	17	7	12	8	52	0.001
Proper poultry house	0	1	1	6	6	14	
Purpose of Poultry Keeping							
Home consumption	18	18	7	9	3	55	
Income	0	1	2	4	5	12	0.01
Both purposes	5	8	4	9	7	33	
The most preferred product							
Eggs	15	18	7	12	2	54	
Meat	3	5	2	5	5	20	0.01
Both products	5	4	4	5	8	26	
System of feeding							
No proper feeding	10	7	5	6	0	28	
Household withdrawal	11	16	6	9	5	47	0.003
Poultry ration	2	4	2	7	10	25	
Proper cleaning of feeders and drinkers							
Yes	5	6	1	8	13	33	0.001
No	18	21	12	14	2	67	

Table 1 (*continued*)

Care and feeding of hens sitting on hatching eggs							
Yes	16	22	11	20	14	83	0.275
No	7	5	2	2	1	17	
Hatchability (%)							
Less than 60	1	2	0	3	0	6	
60- 70	6	6	3	5	4	24	
71- 80	4	8	1	4	6	23	0.551
More than 80	12	11	9	10	5	47	
Vaccination and medication against Diseases							
Yes	4	6	3	9	12	34	0.001
No	19	21	10	13	3	66	
Willingness to vaccinate against Newcastle disease							
Yes	15	22	10	20	15	15	0.05
No	8	5	3	2	0	18	

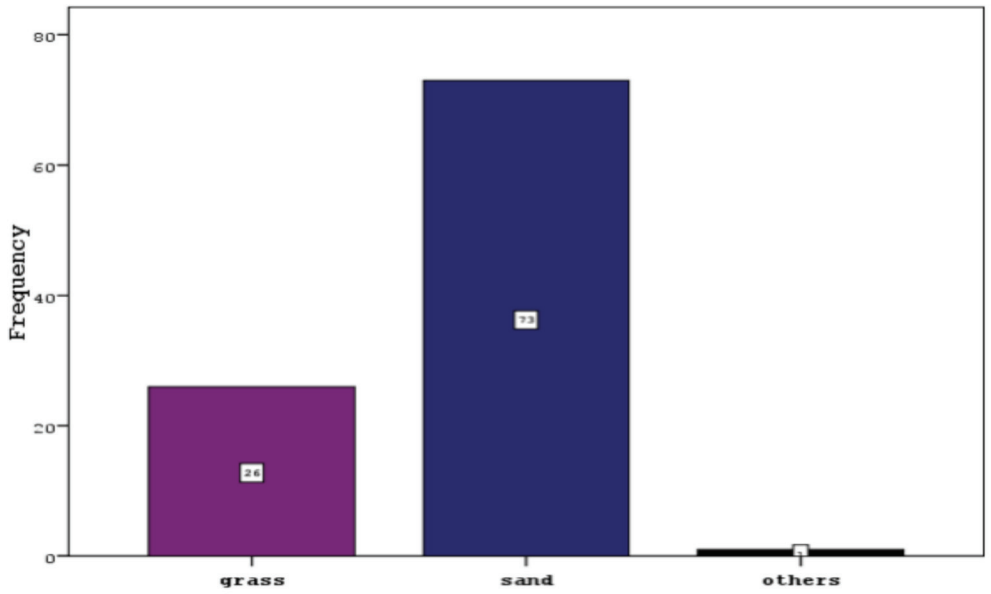

Fig.1: The materials used in the hatching nest

TABLE 2
The effects of flock type on the different managerial aspects of poultry keeping in South Gezira

Parameters	Flock type and breed		Total	Level of significance
	Cross breeds	Local breeds		
Total Flock Size				
Less than 30	0	11	11	0.010
31 – 50	2	21	23	
51 – 70	3	14	17	
More than 70	18	31	49	
System of Housing				0.008
No Housing	3	31	34	
Backyard small pen	13	39	52	
Proper poultry house	7	7	14	
System of Feeding				0.002
No proper feeding	1	27	28	
Household withdrawal and grains	1	36	47	
Commercial poultry ration	11	14	25	
Regular Cleaning of Feeders and Drinkers				0.001
Yes	14	11	25	
No	9	66	75	
The Most Preferred Product				0.786
Eggs	11	43	54	
Meat	5	15	20	
Both products	7	19	26	
Purpose of Poultry Keeping				0.001
Home consumption	5	50	55	
Income	8	4	12	
Both purposes	18	23	33	
Number of Eggs per Clutch				0.455
Less than 10 eggs	0	5	5	
11 – 12 eggs	18	56	74	
More than 12 eggs	5	16	21	
Hatchability Percentage (%)				0.793
Less than 60	2	4	6	
60 – 70	4	2o	24	
71– 80	6	17	23	
More than 80	11	36	47	
Marketing Availability				0.133
Available	6	10	16	
Not available	17	67	84	

Table 2 (*continued*)

Vaccination against Newcastle Disease				
Yes	15	19	34	0.001
No	8	58	66	

TABLE 3
The effects of farmers' gender on the different managerial aspects of poultry keeping in South Gezira

Parameters	Farmers' Gender		Total	Level of significance
	Males	Females		
Flock Type and Breeds				
Cross Breeds	11	12	23	0.178
Local Breeds	25	52	77	
Total Flock Size				
Less than 30	2	9	11	0.472
31 – 50	7	16	23	
51 – 70	7	10	17	
More than 70	20	29	49	
System of Housing				
No Housing	11	23	34	0.745
Backyard small pen	19	39	52	
Proper poultry house	6	8	14	
System of Feeding				
No proper feeding	12	16	28	0.465
Household withdrawal and grains	14	33	47	
Commercial poultry ration	10	15	25	
Regular Cleaning of Feeders and Drinkers				
Yes	28	48	76	0.755
No	8	16	24	
The Most Preferred Product				
Eggs	18	36	54	0.640
Meat	9	11	20	
Both products	9	17	26	
Purpose of Poultry Keeping				
Home consumption	18	37	55	0.745
Income	5	7	12	
Both purposes	13	20	33	

Table 3 (*continued*)

Hatchability Percentage (%)				
Less than 60	3	3	6	0.793
60 – 70	3	21	24	
71- 80	9	14	23	
More than 80	21	26	49	
Vaccination against Newcastle Disease				
Yes	14	20	34	0.439
No	22	44	66	
Willingness to Vaccinate against Disease				
Yes	33	49	82	0.059
No	3	15	18	

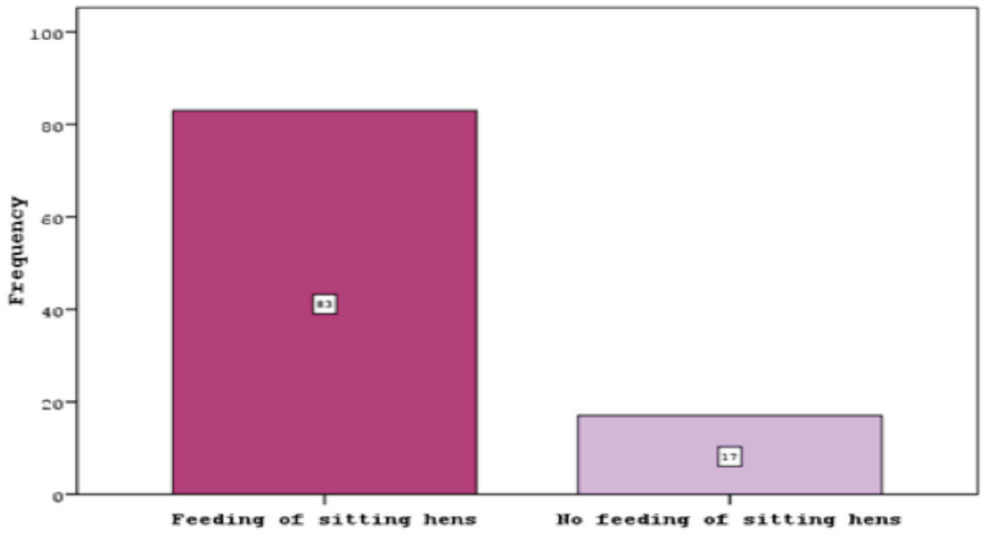

Fig.2: Care and feeding of sitting hens

education between the male and female farmers. Irrespective of the farmers' gender and the flock breeds, more literate farmers (secondary and university) were found to be undertaking good managerial aspects that have positive results on their production (proper poultry houses, poultry rations, big flock sizes, number of eggs per clutch, vaccination and medication against diseases, etc.). Most of the males were shown to keep cross breeds (23 farmers out of 36), and out of this number, 13 farmers had secondary school and university education. When the different managerial aspects were compared with reference to the flock type, the cross breeds significantly obtained the highest value ($P<0.05$) for the best managerial aspect, except for the number of eggs/

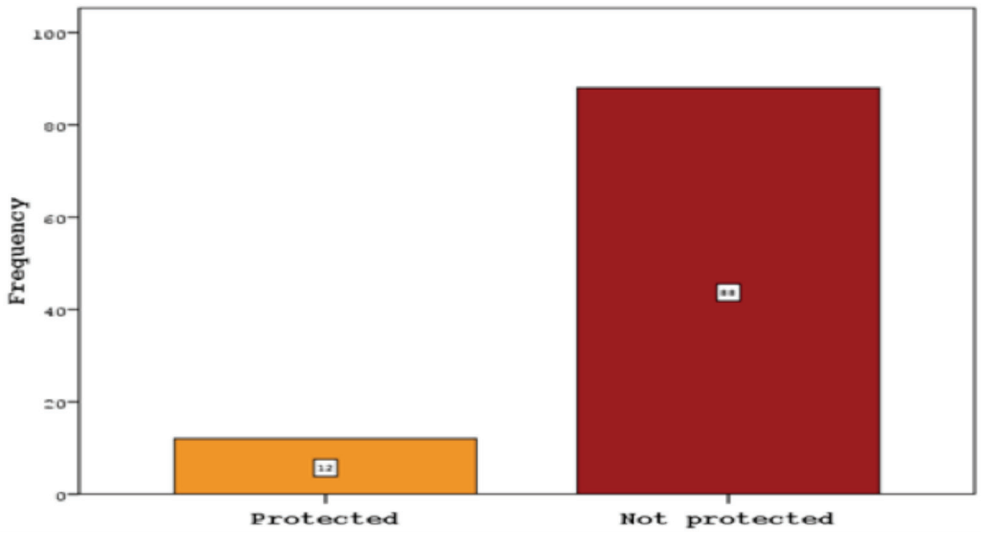

Fig.3: Protection of chicks against environmental conditions

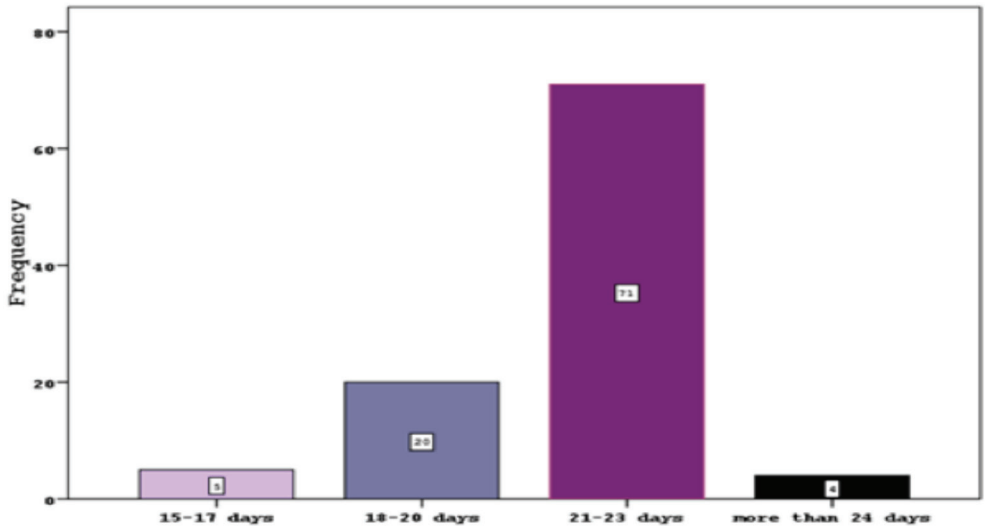

Fig.4: The numbers of days a hen takes in brooding chicks

clutch, which was shown to be fairly better in the cross breeds; inversely, however, the hatchability percentage was found to fairly better for the local breeds (Table 2). Similarly, no significant difference (P>0.05) in term of the managerial aspect (Table 3) was found for another cross tabulation comparison to study the effect of farmers' gender on the different managerial aspects of poultry keeping in this district.

All the farmers agreed that winter is the best season for egg hatching and about 77%

of them used sand (Fig.1); meanwhile, 83% of the farmers stated that they took care of the hens sitting on eggs and fed them (Fig.2). Nevertheless, the majority of the farmers (88%) did not protect the chicks against environmental conditions (Fig.3), which resulted in increasing chick mortality. Most farmers (71%) showed that hens took around 20-21 days in brooding newly hatched chicks (Fig.4).

The results of this study confirm that of Sulieman (1996) who found that the native Baladi hen lays on average of 40–50 eggs per year because there are four clutches of egg laying with an average of 11 eggs per clutches. Under controlled conditions and improved management, however, the average egg production could increase to 172–177 (Sulieman, 1996; Mekki, 1998), and these were apparently attained by more literate farmers who used both proper poultry houses and poultry ration.

Meanwhile, production of eggs for consumption is the principal function of chickens reared in most regions, and these also served as sources of income and meat for home consumption. The production system in all the geographic regions undertaken in the study also revealed similar features that were generally characterized by extensive scavenging management, absence of immunization programs, increased risk of exposure of birds to diseases and predators, and reproduction entirely based on uncontrolled natural mating and hatching of eggs using broody hens. These results are on accord with all the authors reviewed (Host, 1988; Kitalyi, 1998), who had found scavenging fowls as predominating. Housing may not be provided, especially for small size flocks reared by illiterate farmers. These results also confirm those of Huchzermeyer (1973), Kuit *et al.* (1986), Atunbi and Sonaiya (1994) and Illang *et al*. (2000).

The average flock size in this study considered the number of chicks with 20 - 300, and this finding disagrees with that of Khalafalla (2002) who found that the average flock size was 18.8 birds, which included hens (44.3%), cocks (10%), growers (20%) and chicks (24.8%). The hen to cock ratio ranged from 3-6; however, this result coincides with that of Khalafalla (2002) who reported a ratio of 4.4:1.

The remarks and suggestions given by the farmers are summarized as follows:

1. Farmers need packages of poultry keeping.
2. They are looking forward for vaccination against Newcastle disease that is prevailing throughout the year, mainly during the summer, which wipes out more than 90% of their flocks.
3. Farmers complained about the unavailability of the market for them to sale their produce.
4. Some farmers want co-operative societies to help them solve the problems of vaccination and marketing, apart from other constraints that are faced by them.

The major constraints that hinder village poultry production in Sudan have been identified and these included inadequate

health care, poor production, inappropriate housing, as well as poor knowledge of poultry management and poor marketing. In addition, they also do not have access to extension.

CONCLUSION

Based on the results of this study, it is concluded that:

1. Rural poultry production is to be more considered as being an important item in providing animal protein to rural people.
2. Periodic and comprehensive extension packages should be provided to rural poultry keepers so as to cover a more pronounced way of poultry management.
3. Adoption of more research work to find suitable solutions for the constraints that are faced in rural poultry keeping (e.g. housing, feeding, health, hatching egg care, chick brooding and care, vaccination and natural medication).
4. Creation of adequate markets for the farmers to sell their produce, as well as to attract and encourage production of village poultry products.
5. Encourage the establishment of production and consumption co-operatives.
6. Encourage family producers and motivate farmers to become best producers.

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Market Assessment on the Potential of Oil Palm Empty Fruit Bunch (OPEFB) Particleboard in Malaysia's Wood-Based Industries

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ABSTRACT

This study was undertaken to assess the market potential and perceptions of oil palm empty fruit bunch (OPEFB) particleboards in Malaysia's wood-based industries, including furniture. The results of the assessments were based on the reasons and opinions raised by the wood-based product manufacturers and users. The assessment was conducted through a survey which involved 300 respondents during Malaysian International Furniture Fair (MIFF) and Malaysian Furniture Exporters Exhibition (MAFEX) in March 2009 with the aims to investigate the manufacturers' and users' awareness and perceptions towards OPEFB particleboards as an industrial material, to identify OPEFB particleboard potential as an input material and to give general recommendations to OPEFB-based manufacturers, particularly particleboards, so as to help them improving their products based on analysis of the product strengths and weaknesses. The survey data obtained from the fair and exhibition were transformed into tabular and graphical forms. A descriptive analysis was performed for the gathered data to make them interpretable. The 'reasons for choosing' data were analysed with respect the Normality Test by using Kolmogrov-Smirnov Test to determine whether the data are normal or not. The data are considered normal if the mean value is greater than 0.5. The survey showed that most of the respondents preferred rubberwood as a better raw material to be used in producing wood-based products compared to OPEFB. This is because promotion on OPEFB is insufficient and should be done more frequently and widely to gain attentions from wood-based product manufacturers.

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INTRODUCTION

The decrease of forest resources supply is causing concern among wood-based product manufacturers. The industry is therefore encouraged to explore potential resources to ensure continuous supply of raw materials. There are many crops discovered to meet the requirements as alternative materials, such as rubberwood, kenaf, *Acacia mangium* and sesenduk (Izran *et al.,*2009a, 2009b, 2009c, 2009d; Paridah *et al.;* 2009; Khairul *et al.,* 2009). Oil palm empty fruit bunch (OPEFB) was also listed as an alternative material and it has been found to be a good alternative to produce value-added products like particleboards and flat board due to its physical properties and demands, rather than to be utilized as fuel (Nasrin *et al.,* 2008). The utilisation of oil palm biomass in the wood-based industry in Malaysia would help the country to overcome a deficit of 3.85 million m^3 of wood, while strengthening its zero-waste policy between 2006 and 2010 (The Star, 2009). Generally, OPEFB is difficult to be accepted by the manufacturers as a raw material for the production of particleboards due to the great competition from solid wood and several obstacles that need to be overcome. These obstacles include the market coverage and the performances of OPEFB-based products themselves, which are mainly on physical and mechanical properties. In order for OPEFB to be successfully used, the government and private sectors need to focus on research and development (R&D) to reveal its potential as an alternative raw material for solid wood. This should be further supported through the development of the industry in an integrated manner, combining potential manufacturing activities and R&D activities (Ismail *et al.,* 2008). There are many research carried out to study the potential of OPEFB (Mohamad, 1995; Ngan, 2005; Ratnasingam & Wagner, 2009).

Hence, this study focused on one of the obstacles, i.e. market coverage, as information regarding this is very limited for OPEFB. The specific objectives of this study were to: 1) assess the acceptance level of the other alternative materials which are very common in producing wood-based products; 2) investigate the perceptions and awareness of the manufacturers towards OPEFB particleboards as an input for furniture; 3) make general recommendations for the OPEFB particleboard manufacturers to increase their awareness towards their product attributes and characteristics; 4) make recommendations related to environmental issues and consumers' perceptions with regards to OPEFB.

MATERIALS AND METHODS

Determining the Most Accepted Material

This was done to determine the most accepted material among manufacturers in producing wood-based products. It was evaluated based on the niche market. A structured questionnaire was prepared to collect information from furniture buyers and manufacturers who had attended the furniture fairs, i.e. Malaysian International

Furniture Fair (MIFF 2009) and Malaysian Furniture Exporters Exhibition (MAFEX). The respondents were chosen randomly, while the questionnaire was given by hand to the Purchasing and Specification Managers of factories which were also selected randomly to avoid biasness. OPEFB-based samples were also presented to them during the 'questionnaire-answering' session to prevent them from giving bias responses. The samples of OPEFB were distributed based on the final products produced by the factories such as the OPEFB-based furniture for furniture manufacturers.

The Questionnaire

The questionnaire was divided into four parts. The first part covered market segmentation as well as the most-accepted materials (Table 1). There were ten characteristics placed under each subject in this part. The second part covered two subjects, namely, acceptance level of the chosen materials and the reasons for choosing those materials. In determining the most accepted material, parameters evaluated were performances, green based resources, low maintenance, and pollution free, aesthetics, availability, alternative material, quality and cost. Only rubberwood and OPEFB were involved in this evaluation. This is because rubberwood is said to be more popular than other wood species among the manufacturers. In fact, rubberwood is the main raw material used for furniture manufacturing in Malaysia. Based on the current situation, however, rubberwood plantations are slowly being converted into oil palm plantations or housing areas, and a shortage in the rubberwood supply is therefore inevitable. The awareness towards OPEFB as a raw material for particleboard and its properties may have made it suitable as an input material, apart from the availability of the material and the environmental issues in Malaysia's wood-based industries; these were included in the third part of the questionnaire. The fourth section was more on the perceptions and opinions raised on OPEFB particleboard. Each subject included in the second to fourth parts was

TABLE 1
Characteristics under market segments and the most-accepted materials

Market Segments	The Most-Accepted Material
Office Furniture	Rubberwood
Wooden Door	Veneer
Home Furniture	Medum Density Fibreboard
Education Table	LVL
Table Set	Oriented Strand Board
Phone Table	Plywood
Cabinet	Hardwood Lumber
Kitchen	Softwood Lumber
Bedroom Set	Edge-Glued Panel
Others	OPEFB Particleboard

measured using Likert scale (rating scale), ranging from 1 to 5. The rates were ranked as shown in Table 2.

TABLE 2
Likert scale used for ranking (second part of the questionnaire)

Rating	Ranks
1	No Knowledge at all
2	Below Average Knowledge
3	Average Knowledge
4	Above Average Knowledge
5	Perfect Knowledge

The first subject of part three, the scale also ranged from 1 to 5, whereby 1 = Not Aware, 3= Aware, 5= High Level of Awareness. In order to evaluate the other three subjects in part three, the scale was also ranked as 1 = Not Important, 3 = Important, and 5 = Very Important. The respondents were allowed to provide more than one ranking for the last three subjects in part three. Seven characteristics were put under each of the three subjects and only 5 characteristics were given in the last subject, i.e. consumers' perceptions (Table 3).

The survey instrument included some general questions at the beginning of each section, while more specific ones were given at the end of the questionnaire. The questions included one on how to attract the interest of the respondents? In the attempt to maintain the respondents' interest, different forms of questions were incorporated in each part of the questionnaire. Most of the questions had a fixed number of categorical responses, but some were open-ended to allow for opinions to emerge. The questions were open-ended, and this was meant to simplify analysis and coding. A briefing on how to answer the questionnaire was carried out before the respondents began answering the questions and they were also allowed to ask questions if they faced any difficulty in comprehending the questions.

TABLE 3
The Characteristics stated by the four subjects in the questionnaire

Awareness on OPEFB Particleboard	OPEFB Particleboard Attributes and availability	Environmental Issues	People's Perceptions
Furniture	Cost	Green Product	Lack of Information
Door	Surface Uniformity and Smoothness	Recycling Waste	Environmental Friendly
Office furniture	Moisture resistance	Deforestation	Low Cost
Cabinet	Veneer and Laminates Adhesion	Pollution Control	Performances
Chair	Screw and Staple-Holding Ability	Reforestation	Cheaper
Table	Tooling	Food and Fuel	
Kitchen	Porosity	Health and Safety	

Data Analysis

To ensure a better understanding of the data, these were summarized into frequency distributions and presented into tabular and graphical forms. A frequency distribution is a display of occurrence of each score value. It is used to compare the percentages of the proportions of the total number of measurements (Ronald, 1982). Descriptive analysis is one series of nominal values of selection to deflect the real value. It also represents relative percentages to summarize the data so as to make them more interpretable. The tables and graphical charts in the form of Microsoft Excel were used to view the trend in this study. The data for the 'reasons of choosing the material' were analyzed with regards to Normality Test by using One-sample Kolmogorov-Smirnov Test to determine whether or not the data for that subject were normal. The mean value of the data should be more than 0.5 to be considered as normal. Correlation Matrix for OPEFB and particleboards was also conducted. This was an additional analysis done to obtain a matrix giving the correlations between all the pairs of data sets.

RESULTS AND DISCUSSION

Visitors' Reasons for Visiting the Fairs

Based on the analysis, 95% of the 300 respondents attended the fairs were manufacturers and 5% were users. Forty seven percent of the manufacturers were from wood-based industries, particularly the panel industries, and the remaining (53%) were furniture manufacturers. Twenty one percent of the manufacturers were among the participants. 300 respondents gave different reasons for attending the fairs, as presented in Table 4.

TABLE 4
Respondents' reasons of attending the fairs

Reasons	Percentage (%)
To know new products in the market	29.8
To place orders	20.23
To build connection and visit suppliers	19.6
Gathering current furniture industry status	14.9
Business opportunity	12.39
To seek representatives for the factories	3.89

The Most Accepted Material and Market Segment

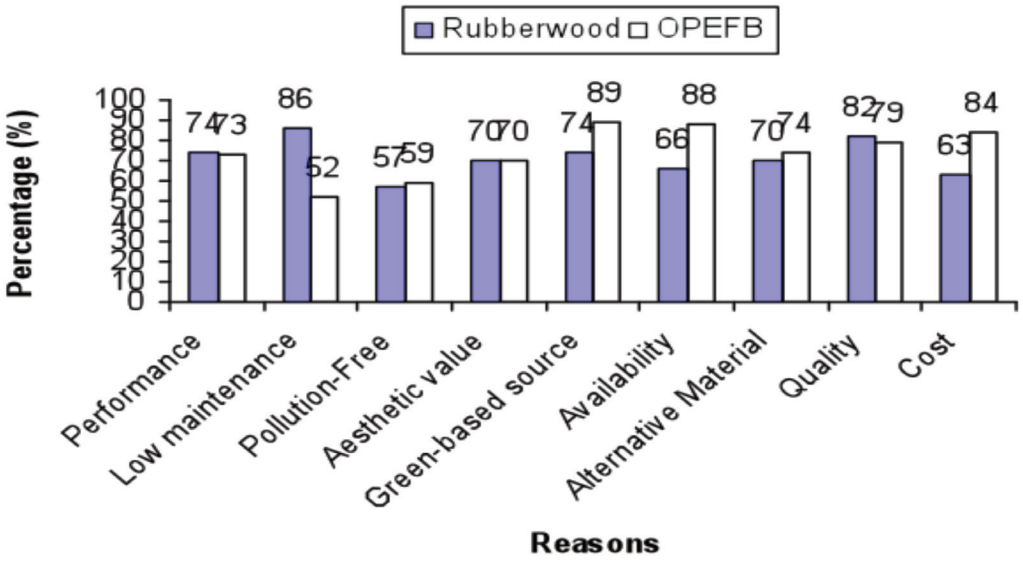
Based on the analysis of the questionnaire, 97% of the manufacturers chose rubberwood as the most accepted material for producing wood-based products. This indicated that majority of the manufacturers chose rubberwood as the best raw material. The other group of manufacturers agreed that *jelutong* and *sesenduk* are better as input materials for wood-based products. As rubberwood was found to be the most accepted material, it was therefore chosen to be compared with OPEFB in the 'reasons of acceptance' analysis. The results of the comparison are illustrated Figure 1. Surprisingly, OPEFB was found to be superior to rubberwood in terms of cost-effectiveness, good alternative material,

availability, better green-based source, and pollution-free. OPEFB was found to have a similar value with rubberwood for being chosen due to its aesthetic value. Rubberwood, on the other hand, was found incomparable for its performance, maintenance ability and quality. This analysis clearly showed greater confidence obtained by OPEFB from the manufacturers as compared to rubberwood. This finding further leads to the following question: If the manufacturers are very confident with OPEFB, why do they still prefer rubberwood as a raw material for their products? The answer to this question can be found under the 'market segment' section, which explains the influence of demand.

Market Segment

The market segment was dominated by home furniture sector, followed by office furniture, table set, bed room set, education table, cabinet, kitchen, phone table, wooden door, and others. The percentage of each market segment is exhibited in Fig.2.

The percentage of the market segment also explains the pattern in the demands for those wood-based products. This means a higher percentage of the market segment indicates a higher demand. Based on the data given in Fig.1, home furniture receives the highest demand, which indirectly encourages manufacturers to utilize the accepted alternative raw materials for producing home furniture. This is probably the reason for the higher acceptance level



*The mean values for each parameter of each raw material were found insignificant

Fig.1: Acceptance reasons for rubberwood and OPEFB.

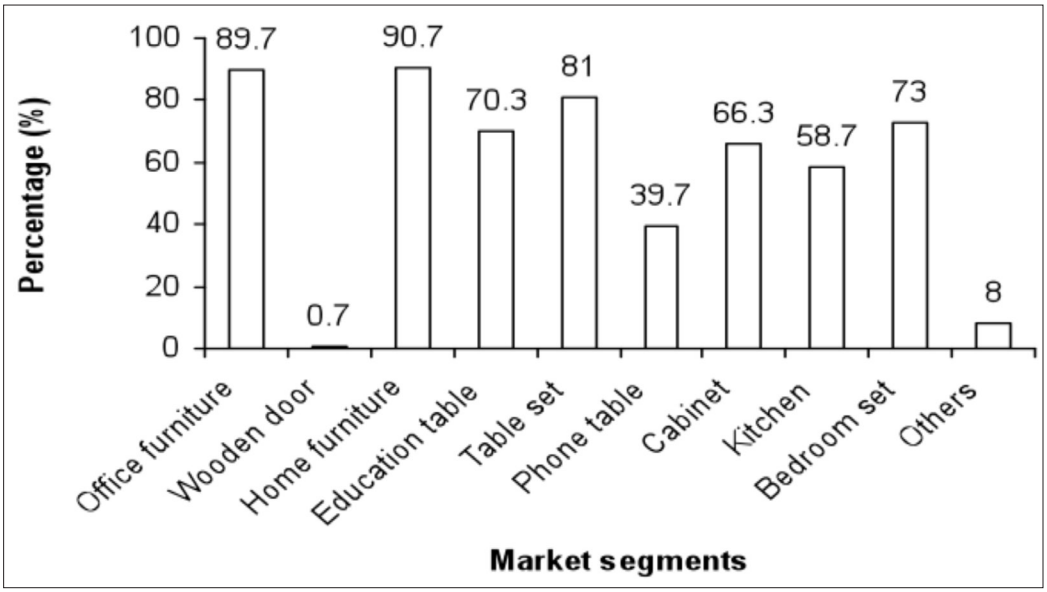


Fig.2: Percentage of the market segments

for rubberwood compared to OPEFB, even though OPEFB has greater manufacturers' confidence (refer to the 'reasons of acceptance').

Awareness towards OPEFB Particleboard

In response to the awareness towards OPEFB particleboard, the manufacturers were found be aware of the existence of this particular material in the industry. According to them, OPEFB particleboard is available and gradually replacing rubberwood-based particleboard as an alternative raw material for the production of wood-based products. This is probably due to the demand of rubberwood that has overshadowed its supply and caused its price to increase and eventually burdened most manufacturers and encouraged the manufacturers to switch to OPEFB (Paridah *et al.,* 2009). The manufacturers aware that OPEFB particleboard is frequently used for production of home furniture (awareness score: 4.9), followed by office furniture (awareness score: 4.7), cabinets (awareness score: 4.7), tables and kitchens (awareness score: 4.5), chairs (4.4) and doors (3.1). Once again, the manufacturers agreed that the awareness level was influenced by the consumers' demands.

Preferred Particleboard Attributes

Before promoting the use of OPEFB particleboards among manufacturers, it is crucial to know the kind of particleboard attributes that are preferred by manufacturers and customers (refer to Table 3, under OPEFB Particleboard Attributes and availability). As shown, there were insignificant differences between the parameters studied. This further indicates that all the presented attributes are very

TABLE 5
Correlation Matrix of the Characteristics of Rubberwood Particleboard

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
A1	1.00									
A2	0.09	1.00								
A3	0.02	-0.22	1.00							
A4	0.01	0.39	-0.04	1.00						
A5	-0.05	-0.20	0.03	-0.33	1.00					
A6	0.07	-0.17	0.15	0.02	-0.13	1.00				
A7	-0.07	-0.12	-0.15	-0.18	-0.08	-0.26	1.00			
A8	0.14	0.53	-0.07	0.19	-0.12	-0.10	-0.18	1.00		
A9	-0.07	-0.24	-0.02	-0.08	0.01	-0.04	0.04	-0.39	1.00	
A10	0.01	0.41	-0.01	0.12	-0.14	-0.05	-0.07	0.21	-0.24	1.00

A1: Acceptance Level, A2: Performances, A3: Low Maintenance, A4: Pollution Free, A5: Aesthetics, A6: Green Based Resources, A7: Availability, A8: Alternative Material, A9: Quality, and A10: Cost

TABLE 6
Correlation Matrix of the Characteristics for OPEFB Particleboard

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
A1	1.00									
A2	-0.02	1.00								
A3	0.02	-0.40	1.00							
A4	0.06	-0.12	-0.11	1.00						
A5	-0.07	0.01	-0.08	-0.21	1.00					
A6	0.10	-0.09	0.02	-0.10	0.02	1.00				
A7	0.09	-0.17	0.09	-0.11	-0.01	-0.03	1.00			
A8	-0.09	-0.05	-0.11	-0.19	0.83	0.09	-0.02	1.00		
A9	-0.09	0.18	-0.04	-0.20	0.03	-0.16	-0.14	0.01	1.00	
A10	0.08	-0.07	0.04	0.07	-0.23	-0.01	0.04	-0.26	-0.16	1.00

A1: Acceptance Level, A2: Performances, A3: Low Maintenance, A4: Pollution Free, A5: Aesthetics, A6: Green Based Resources, A7: Availability, A8: Alternative Material, A9: Quality, and A10: Cost.

important for the manufacturers. The ratings of the attributes were cost (4.52), tooling (4.45), aesthetic value (4.44), porosity (4.39), surface smoothness and uniformity (4.35), veneer and laminate adhesions (4.34), moisture resistance (4.31) and screw and staple holding ability (4.30). Cost is shown to be the most important attribute in producing particleboard. Therefore, to make OPEFB one of the favourite materials among the manufacturers, it has to fulfil all the attributes rated by them. Unfortunately, like other materials, OPEFB also possesses certain weaknesses. According to the manufacturers, OPEFB particleboards have poor surface uniformity and smoothness,

low moisture resistance that causes fibre to swell and destroy paints, veneers and laminates do not adhere properly, low screw and staple-holding ability and are too hard on tooling. Nonetheless, they also agreed that OPEFB particleboards are much more cost effective than rubberwood-based particleboards.

Environmental Issues

Some important environmental issues that have encouraged the manufacturers to switch to alternative materials (instead of using solid wood of natural forests) are presented in Fig.3.

From the figure, three top environmental issues for the manufacturers were searching for green products (98.3), reforestation (96.3) and the use of waste (95.7). Based on the characteristics of the OPEFB, this material can be undoubtedly declared as an 'environmental-friendly' material. OPEFB is abundantly available as a waste which can be fully utilized for substituting solid wood, and this may help in reducing the heavy reliance on natural forests for continuous supplies of raw materials for the industry. The advantage of the OPEFB may also fulfil the requirements for it to be awarded as a green-product. If these are the advantages that the manufacturers are looking for, then OPEFB particleboards will definitely be preferred, and this can thus enhance the opportunity of OPEFB particleboards to penetrate the wood-based products market.

Respondents' Perception of OPEFB Particleboards

The highest rated perception towards OPEFB particleboard was the 'lack of information about the panel' (98.6%). Thus, to form good market coverage for OPEFB particleboards, it is essential to change this perception. Users' and manufacturers'

TABLE 7
Mean and Standard Deviations of the Characteristics of Rubberwood Particleboard

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Mean	4.90	0.74	0.86	0.57	0.70	0.74	0.66	0.46	0.82	0.63
SD	0.35	0.44	0.35	0.50	0.46	0.44	0.47	0.50	0.39	0.48

A1: Acceptance Level, A2: Performances, A3: Low Maintenance, A4: Pollution Free, A5: Aesthetics, A6: Green Based Resources, A7: Availability, A8: Alternative Material, A9: Quality, and A10: Cost

TABLE 8
Mean and Standard Deviations of the Characteristics of OPEFB Particleboard

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Mean	3.64	0.73	0.52	0.59	0.70	0.89	0.88	0.74	0.79	0.84
SD	0.89	0.44	0.50	0.49	0.46	0.31	0.32	0.44	0.41	0.37

A1: Acceptance Level, A2: Performances, A3: Low Maintenance, A4: Pollution Free, A5: Aesthetics, A6: Green Based Resources, A7: Availability, A8: Alternative Material, A9: Quality, and A10: Cost

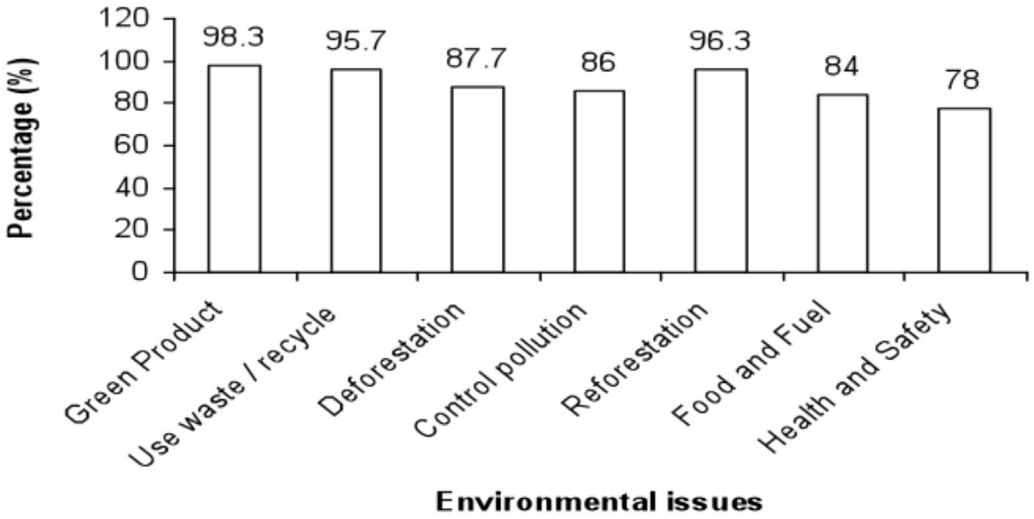


Fig.3: Environmental Issues

lack of information may limit the use of OPEFB particleboards. Hence, promotions introducing OPEFB particleboards should be carried out as soon as possible, and this needs to be done while the manufacturers are still searching for alternative materials with the intention to replace solid wood. The second highest rated perception was that OPEFB particleboards are much cheaper (97.7%) and more environmental-friendly (95.3%) than wood-based particleboards. It is because OPEFB particleboards are fabricated from wastes, which involve no high cost and are also environmental-friendly. These perceptions seem to provide a good chance to form a market for OPEFB particleboards as most manufacturers are looking for cost effective material (refer to the preferred particleboard attributes) and green product (refer to environmental issues) for their productions. Seventy six percent and seventy one percent of the manufacturers felt that OPEFB incurred low cost and possessed good physical and mechanical performances. Some manufacturers, however, thought that OPEFB was not suitable to be used in furniture and household applications due to its bad performances and low cost compared to the conventional particleboards.

Therefore, among the suggestions that can be given to the manufacturers to help them improve their OPEFB-based products and form a wide market are: (1) OPEFB particleboard manufacturers must address some of the major technical problems mentioned by many of the respondents (such as surface smoothness, aesthetics, moisture, etc.) prior to entering the competitive furniture and door markets; (2) OPEFB particleboard manufacturers should emphasize the product's advantages (namely, strength, dimensional stability, etc.), and ensure that all consumers are

aware of these by having brochures and fact sheets in furniture and door outlets.

Correlation Matrix for Particleboards

Tables 5 and 6 show the correlation matrix for the characteristics of rubberwood particleboard and OPEFB particleboard manufacturers, respectively. The correlation coefficient values were found to range between -0.39 and 0.53 for rubberwood particleboards, and -0.40 and 0.83 for OPEFB particleboards. The highest correlation in the matrix for rubberwood was 0.53, between the characteristics of the performances and alternative materials, while the lowest was -0.39, which was between the characteristics of the alternative materials and quality. For OPEFB particleboards, the highest correlation in the matrix was 0.83, i.e. between the characteristics of aesthetics and alternative materials, while the lowest was -0.40, i.e. between the characteristics performances and low maintenance. These correlations were strong enough to justify the analysis.

On the contrary, the correlations between the variables of low maintenances, pollution free, quality and cost were found to be negative, while all the other correlations were positive for OPEFB particleboards. These correlations show that most manufacturers disagreed with these statements.

Tables 7 and 8 reveal that the means of most characteristics were quite low for rubberwood particleboards and OPEFB particleboards, suggesting that in most cases, the respondents have accepted particleboards as a premier furniture material. In particular, the respondents mostly disagreed in term of the acceptance level of the OPEFB particleboard characteristics, based on their previous research and knowledge. The deviation in the characteristics indicated a general consensus among the consumers. The lowest value of deviation for this study was performance, and this indicated that consumers strongly agreed that OPEFB particleboard was a good material for furniture and doors. According to Ratnasingam and Wagner (2009), people strongly agreed that particleboard-based furniture is perceived to incur low cost, but consumers disagreed on other characteristics such as its machining, attractiveness, dimensional stability, uniform thickness and warp free.

CONCLUSIONS

The findings of this study have clearly revealed that manufacturers and users (respondents) of wood-based products have various opinions towards the alternative material that has recently been introduced with intention to reduce the usage of solid wood, which may eventually reduce deforestation. Most of the respondents still preferred rubberwood as the best raw material for the production of wood-based products due to its acceptable physical and mechanical properties. Meanwhile, OPEFB is still in the initial stage of gaining manufacturers' confidence but is in its way to overtake the popularity of rubberwood. Various promotions on OPEFB as an input for wood-based products should be seriously

done in order to increase its acceptance level among the respondents as they were actually not aware with the existence of the material. The main reasons of the low acceptance level indicated for OPEFB and OPEFB particleboards were the lack of information about the material and products. OPEFB particleboard is presently utilized as a non-used material in the manufacturing industry. In order to penetrate market places, specific target markets and technical strategies must be undertaken.

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Nephrotoxicity and Hepatotoxicity Evaluation in Wistar Albino Rats Exposed to *Nauclea latifolia* Leaf Extracts

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ABSTRACT

Consumption of the aqueous leaf extract of *Nauclea latifolia* as anti-malaria concoction without any recourse or regard for its safety is a common practice in the Northern Nigeria. The aim of this study was to evaluate the safety efficacies of the ingestion of the methanolic leaf extract of this plant on the liver and kidney functions in wistar albino rats. Acute toxicity tests were carried out to determine LD_{50}, while sub-chronic toxicity study was carried out by oral administration of graded doses (200, 400, 800, 1600 and 3200mg/ Kg) of the extract to different groups of rats for 30 days. Both the liver and kidney functions assessed biochemically using standard methods revealed the LD_{50} of *N. latifolia* at 3200mg/Kg body weight as being non-lethal. Meanwhile, biochemical and histological results obtained for the liver and kidney function parameters indicated that ingestion of *N. latifolia* leaf extract has no observable toxic effects on these organs at the tested doses. It was therefore suggested that these results could form the basis for clinical trial in human.

Keywords: Hepatotoxicity, *Nauclea latifolia* Nephrotoxicity, wistar albino rats

INTRODUCTION

Medicinal plants have been known to be useful in the treatment of various diseases all over the world since the time immemorial. In addition, plants derived products have been used for medicinal purposes for centuries. In fact, it was estimated that about 80% of the world population rely on botanical preparations as medicines to meet their health needs (Shri, 2003). The uses, modern applications and general therapeutic claims of these herbs receive widespread attention, not only in Nigeria but worldwide day by day (Jyoti *et al*., 2009). Apart from

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the documented severe toxic reactions arising from the use of herbs, general public and professional traditional medical practitioners/healers sometimes mistakenly think of herbs as natural alternative to drugs, failing to recognize/realize that herbs contain bioactive chemicals, some of which may be toxic (Tyler, 1994). However, many patients are under false assumption that naturally derived herbal medicines are safer with fewer side effects but this is not totally true (Gamaniel, 2000).

Nauclea latifolia (*Rubiaceae*) is a tree species grown in the northern parts of Nigeria, commonly known as "Tuwonbiri"or "Tafashiya" in Hausa, "Ubulumu" in Igbo and "Opepe" in Yoruba, has been claimed to be valuable in a wide spectrum of ailments (Onyeyili *et al*. 2001; Ajagbonna *et al*., 2002). Nworgu *et al*. (2008) reported blood pressure lowering effect of *N. latifolia* in rats, while potential anti-diabetic properties of the plant were recorded by Gidado *et al*. (2005). Many people in Northern Nigeria treat malaria by drinking aqueous leaf extracts of *N. latifolia*; however, the responses of various organs, especially the liver and kidney (sites of biotransformation) in humans to ingestion of this extract, remain scientifically unknown. More so, there was little or dearth of information on the effects of the extract of this plant at the cellular level. Thus, this study was undertaken to examine to what extent the liver and kidney would be affected in rats exposed to *N. latifolia* leaf extract.

MATERIALS AND METHODS

Plant Materials and Preparation of Plant Extracts

The leaves of *N. latifolia* were collected within Sokoto metropolis and authenticated at the Biological Sciences Department, University of Agriculture, Abeokuta by Dr. Aworinde D.O. (Plant Taxonomist/ Anatomist). The leaves were washed with tap water, air-dried and pulverized using a grinding machine. Three hundred grams (300g) of the ground sample was immersed in absolute methanol (1000ml) for 72 hours, under rigorous shaking/mixing to ensure maximum extraction. The extract was filtered through Whatman filter paper No 1, and the decoction was concentrated to dryness in rotary evaporator to obtain the crude methanolic extract, which was stored in a refrigerator until used. The extract yield was 9.8% of the starting materials.

Phytochemical Screening of the Aqueous and Methanolic Extracts of Nauclea latifolia

Phytochemical screening was carried out according to the methods proposed by Trease and Evans (1978), as described by Edeoga *et al*. (2005).

Test for tannins: The dried powdered leaf (0.5g) was boiled in 20ml of water in a test tube and then filtered. Two drops of 0.1% (w/v) ferric chloride reagent were added and observed for brownish-green or brownish-green to indicate the presence of tannins.

Test for saponins: Two (2) grams of powdered leaf was boiled in 20ml of distilled water in water bath and filtered. 10ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously for stable persistence froth. The frothing was mixed with 3 drops of olive oil, before it was shaken vigorously and observed for the formation of emulsion.

Test for flavonoids: Powdered leaf (5mg) was heated in 10ml of ethylacetate over a steam bath for 3 min. The mixture was filtered and 4ml of filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration was observed, indicating a positive test for flavonoid.

Test for steroid: Acetic anhydride (0.2ml) was added to 0.5g methanolic extract of each sample with 2ml H_2SO_4. The colour was expected to change from violet to blue or green.

Test for terpenoids (Salkowski test): The extract (0.5g) was mixed with 2ml chloroform and concentrated H_2SO_4 (3ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive result for the presence of terpenoids.

Test for cardiac glycosides (Keller Killani test): Methanolic extract (0.5g) was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1ml of conc. H_2SO_4. A brown ring of the interface indicates a deoxy sugar that characterized a cardenolides. A violet ring appeared below the brown ring, while in the acetic acid layer, a greenish ring was gradually formed throughout thin layer.

Experimental Animals

Thirty six (36) Wistar albino rats (weighing 180-240g) of both sexes, obtained from the Department of Veterinary Anatomy, University of Ibadan, were used for the study. They were housed in well-ventilated rat cages, kept at 27-30^{o}C, with 12 hour natural light and 12 hour darkness, and allowed free access to tap-water and dried rat pellets (Ladokun & Sons Feeds, Ltd). They were also allowed to acclimatize for a week before the commencement of the experiment.

LD_{50} Determination/Acute Oral Toxicity Study

This was carried out according to the procedure described by Oduola *et al.* (2010). Briefly, graded doses of the extract were administered orally to six (6) groups of rats consisting of six (6) rats per group. Thus, Group 1 served as a control and received normal saline, while Groups 2, 3, 4, 5, and 6 received 200, 400, 800, 1600 and 3200 mg/Kg body weight respectively, with the aid of canula attached to a graduated syringe. All the rats were placed under observation for 24 hours, after which the number of dead rats was recorded and LD_{50} was calculated using the formula described by Aliu and Nwude (1982).

Sub-chronic Toxicity Study

After 72 hours, none of the rats in oral toxicity study died. Thus, the extract was administered to the animals for 30 more days, at the end of which, the rats were weighed, and their blood samples were

collected through cardiac puncture under chloroform anesthesia into lithium-heparin specimen bottles for biochemical assays. The rats were then sacrificed by cervical dislocation, while liver and kidney collected for function tests and histopathological examinations were carried out using the standard techniques.

All the biochemical parameters were determined using the Chromatest reagents diagnostic kits, except for Glutathione-S-transferase (GST) whose activity was determined using the method of Habig and Jakoby (1980).

Histopathological Studies

The histological examinations of the liver section of the representative samples of these groups of rats were carried out following standard procedures.

Statistical Analysis

The mean, standard deviation and level of significance for the difference between the means of the data generated were computed using student test SPSS 6.

RESULTS

The present study attempted to evaluate the effects of ingestion of the methanolic leaf extract of the plant *Nauclea latifolia* on the liver and kidney functions in wistar albino rats. The results of the phytochemical screening of the aqueous and methanolic extracts of *N. latifolia* are presented in Table 1.

TABLE 1
The results for the Phytochemical constituents

Tests	Results	
	Methanolic extract	Aqueous extract
Cardiac glycosides	++	+
Flavonoids	++	+
Saponins	+	+
Steroids	-	-
Terpenoid	+	+
Tannins	+	+

++ Highly present
+ Present
Absent.

The results of the acute oral toxicity study revealed that there was no record of death even at the highest dose of 3200mg/

TABLE 2
LD_{50} estimation by arithmetic method adapted by Aliu and Nwude (1982)

Dose (mg/Kg)	No. of rats	Death	Dose Difference	Mean Death	Dose Difference *Mean Death
Saline	6	0	0	0	-
200	6	0	200	0	-
400	6	0	400	0	-
800	6	0	800	0	-
1600	6	0	1600	0	-
3200	6	0	3200	0	-

Kg b.wt, as shown in Table 2. This indicated that the LD_{50} of the plant was higher than 3200mg/Kg. In fact, all the animals appeared healthy and active throughout the experiment.

Table 3 shows the values of some electrolytes (Na^+, K^+ and HCO_3^-), urea and creatinine levels between the control and the studied groups, as well as between the groups. Table 4 presents the values of total protein and albumin, ALT, AST, ALP and GST obtained for the control and studied groups (B, C, D, E and F). The plant extract over the range of tested doses showed very insignificant changes rather than producing toxicity as compared to normal.

DISCUSSION

Liver and the kidney play important roles in the biotransformation of the ingested. In particular, the liver is much more prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism, its portal location within the circulation and its anatomic-physiologic structure. The kidney, on the other hand, is highly susceptible to toxicants because of a high volume of blood flows through it and it also filters large number of toxins which can concentrate in the tubules. The leaf extract *N. latifolia* was found to contain high level of cardiac glycoside, moderate levels of flavonoids, saponins, terpenoid and tannins. However, steroids were not present in the tested doses. Judging by the current means of estimating the current LD_{50} values, based on acute oral toxicity recommended by the Global Harmonised Systems of classification and labelling of chemicals on toxicants (Link/URL, 2010), the LD_{50} for this particular plant extract would be greater than 3200mg; this suggests or implies that the extract is non-lethal at 3200mg, and it is therefore assumed to be safe for consumption.

TABLE 3
Effects of intake of *Nauclea latifolia* methanolic leaf extract on kidney function

Groups / Parameters	Group 1 (control)	Group 2 (200mg/Kg)	Group 3 (400mg/Kg)	Group 4 (800mg/Kg)	Group 5 (1600mg/Kg)	Group 6 (3200mg/Kg)
Sodium(mmol/l)	125 ± 3.75	124 ± 4.32	127 ± 5.24	126 ± 3.45	125 ± 5.10	122 ± 4.13
Potassium (mmol/l)	4.58 ± 0.88	4.42 ± 0.76	4.38 ± 0.93	5.43 ± 0.47	5.23 ± 0.81	4.34 ± 0.62
Bicarbonate(mmol/l)	26.17 ± 2.1	24.66 ± 2.81	25.17 ± 1.31	27.17 ± 1.8	25.07 ± 1.10	22.71 ± 3.40
Chloride (mmol/l)	111.83±9.4	109.28 ± 5.68	108.19±9.30	103.50 ± 5.4	105.25 ± 5.40	107.33 ± 6.21
Urea (mmol/l)	6.71 ± 1.50	6.30 ± 1.81	6.32 ± 1.64	6.52 ± 0.99	6.04 ± 0.98	6.21 ± 0.65
Creatinine (mmol/l)	89.97±5.71	90.19 ± 14.33	93.11±10.36	90.34±12.61	92.11 ± 10.30	90.38 ± 8.21

TABLE 4
Effect of intake of *Nauclea latifolia* methanolic extract on liver function profiles

Groups / Parameters	Group 1 (control)	Group 2 (200mg/Kg)	Group 3 (400mg/kg)	Group 4 (800mg/kg)	Group 5 (1600mg/kg)	Group 6 (3200mg/kg)
Total Proteins (mg/L)	3.42±0.31	3.85±0.32	3.91±0.29	3.66±0.33	3.67±0.31	3.33±0.40
Albumin (mg/L)	1.30±0.07	1.43±0.07	1.45±0.11	1.34±0.08	1.42±0.07	1.36±0.08
Total Bilirubin (µmol/L)	10.5±2.10	7.65±2.02	8.76±2.03	8.33±2.01	8.45±2.11	8.14±2.30
Conj. Bilirubin (µmol/L)	2.77±0.16	2.63±0.36	2.54±0.16	2.67±0.75	2.70±0.12	2.40±0.34
ALT (1 U/L)	25.32±1.30	26.38±1.28	26.67±1.40	27.14±1.61	27.83±2.01	25.19±2.34
AST (1 U/L)	20.17±1.29	21.88±1.26	21.64±1.70	21.28±2.00	20.71±2.11	20.86±1.44
ALP (1 U/L)	50.34±5.00	51.07±5.10	52.22±4.20	51.98±4.20	53.06±4.46	53.18±4.31
GST(units/mg)	0.96±0.04	0.99±0.03	0.94±0.02	0.90±0.04	0.82±0.03	1.12±0.41

The observed non-toxic effect or the absence of hepatocellular or nephrotoxical damage at these investigated concentrations could be buttressed by the non-significant differences in the liver and the kidney function parameters, which revealed that the conjugating ability of the liver was not compromised, especially from the total and conjugating bilirubin levels obtained. Meanwhile, non-hepatocellular damage as revealed by the ALT and AST values which were further buffered by the histological revelation. The liver sections of the control and the tested groups showed no gross lesion, except for mild hepatic vacuolation which had been observed at 3200mg.

The incidence of nephrotoxicity was also determined as a marker level of the kidney function (electrolytes, urea and creatinine) in all the experimental animals and control within the reference range throughout the period of the study. This is in agreement with the report of Ajagbonna *et al*. (2002) and also the traditional belief that the consumption of *N. latifolia* aqueous extract, as an anti-malaria agent, seems to be not imposing any serious harmful effect(s). The results are also in agreement with the report of Mesia *et al*. (2005) who stated that the *N. latifolia* extract posed no toxicological threat to the consumer when administered as traditional remedies for malaria.

Meanwhile, the histopathological examinations of the kidney in the control and treated rats showed no visible lesion or necrotic sign. The results of this study suggest that ingestion of *N. latifolia* (at the tested concentration) has no adverse effect on the liver and kidney functions in rats. Therefore, the present study has established that ingestion of *N. latifolia* extract has no observable adverse effect(s) on the liver and kidney of rats and this could form a basis for

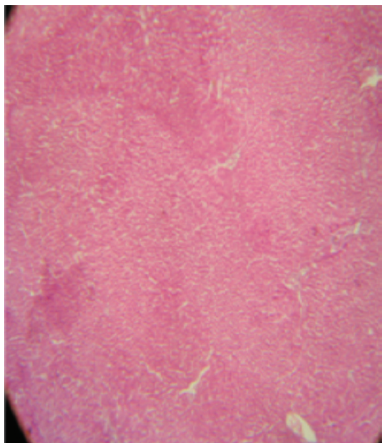

Fig.1: Section of the liver tissue showing normal hepatocytes (control, X400 magnification)

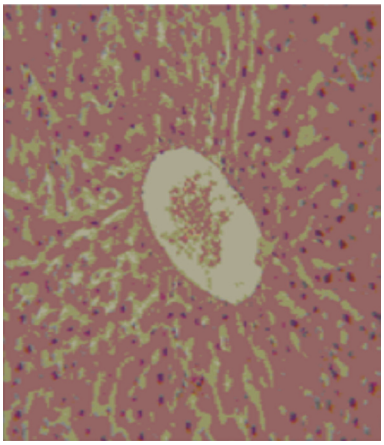

Fig. 2: Liver section showing no visible lesion (Group 2, X400 magnification)

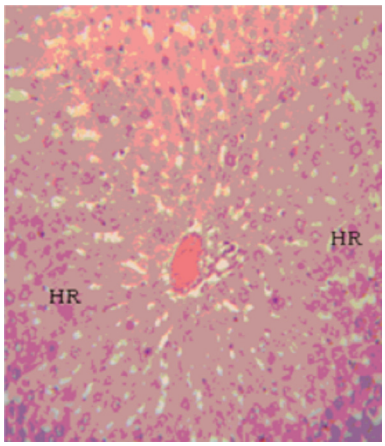

Fig. 3: Liver section showing hepatic regeneration (HR) without visible pathology (Group 3, X100 magnification)

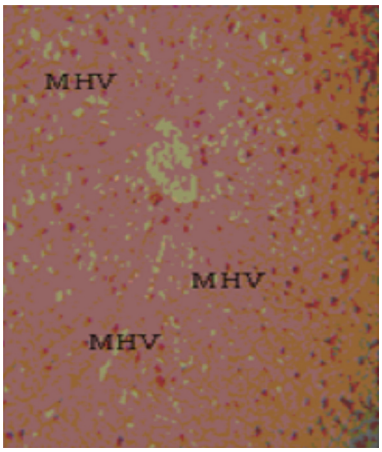

Fig. 4: Liver section showing mild heptic vacoulation (MHV) (Group 6, X100 magnification)

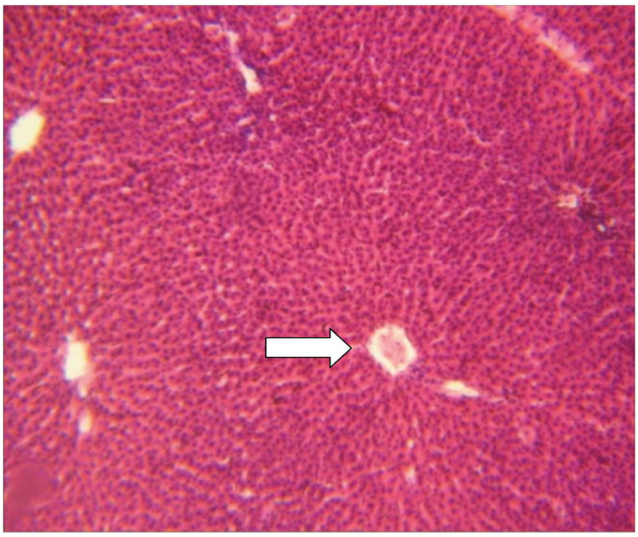

Fig.5: Liver section showing normal portal tract (group 5) x 100

the clinical trial in human. In conclusion, it can be concluded that *N. latifolia* has potential to be used in the management of hepatic and nephritic damages.

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Malaysian Consumers' Preference and Willingness to Pay for Environmentally Certified Wooden Household Furniture

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ABSTRACT

Demand for certified timber products (CTPs) is on the rise, with major markets currently in North America and Europe, where consumers are willing to pay price premiums for these wood products. It is reported that there is little or no local demand for CTPs in the developing producer countries as consumers are said to have little interest in the products and cannot afford to be environmentally ethical in their consumption. A survey was conducted in Kuala Lumpur to determine whether consumers in Malaysia, which is a tropical CTPs producing and exporting country, have a preference and willing to pay price premiums for environmentally certified wooden household furniture (ECWHF). The willingness to pay (WTP) was estimated with the contingent valuation method using the Turnbull lower-bound estimator. The results indicated that a majority (74%) of the respondents showed a preference for ECWHF when priced at similar bid level with its identical non-certified products. However, a much lower percentage of these respondents were found to be willing to pay a price premium for the products. Of the 994 respondents surveyed, only 40.7% indicated a positive WTP. On average, the respondents were willing to pay about 18% more for ECWHF over its identical non-certified competitor. CTPs may be appropriate for specific niche markets which should be identified by marketers of these wood products.

Keywords: Contingent valuation, consumer, preference, willingness to pay, certified timber products

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INTRODUCTION

At the Rio de Janeiro's Earth Summit in 1992, it was agreed that the world's forests are to be sustainably managed and wood products entering the international trade should originate from areas that are certified

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to practise sustainable forest management (SFM). Since then, many initiatives have been formulated and done to address and implement forest management and timber product (or chain-of-custody) certification schemes. At the moment, about 356.7 million ha of forests (approximately 9.0% of the world's forests) have been certified under various certification schemes worldwide (UNECE, 2010). It was estimated that about 471.8 million m^3 of industrial roundwood could be produced from these certified forests, representing about 26.4% of the world's industrial roundwood production.

One of the major issues in marketing certified timber products (CTPs) to consumers is their willingness to pay price premiums as these wood products are expected to be more expensive than non-certified timber products (Jensen *et al*., 2004). This is because sustainable forest management and certifying the practice are expected to cost more than the present forest management practices (Fischer *et al*., 2005; Leslie, 2006; Chen *et al*., 2010). On average, the total costs for introducing a forest management certification system and implementing higher management standards could cause forest management costs to increase by 5% to 25% (Nussbaum *et al*., 1996; Sikod, 1996; Williams *et al*., 1997). Abdul Rahim (2002) reported that the compliance with SFM practices has imposed an incremental cost of about 69.6% to logging concessions in Malaysia. Meanwhile, the total harvesting cost under the SFM was estimated at RM198.54/m^3 compared to merely RM117.03/m^3 using the conventional logging method. In addition, the subsequent chain-of-custody certification would add more cost in making CTPs available to the customers. These increases in cost are expected to be passed on to consumers in the form of more expensive CTPs. It is, however, believed that consumers would be willing to pay more for products originating from certified, sustainable managed forests (Merry & Carter, 1997), with a premium ranging from 5% to 10% (Forsyth, 1998).

Demand for certified timber products, both in the business and consumer markets, is reported to be on the rise (Jayasinghe *et al*., 2007). The market for CTPs is currently in North America and Europe (Durst *et al*., 2006), where consumers are said to be more concerned with the environmental impacts of the products they purchase (Rowlands *et al*., 2002; Moon & Balasubramaniam, 2003). An increasingly large number of individuals in these markets are willing to pay price premiums for environment-friendly products (Laroche *et al*., 2001). Homeowners in the USA, for example, are willing to pay an average of 12.5% more for environmentally certified wood products (Ozanne & Vlosky, 1997). A more recent study by Aguilar and Vlosky (2007) reported that consumers in the USA are willing to pay between 10% and 25% more for CTPs. Veisten (2007) estimated the willingness to pay (WTP) for eco-labelled wooden furniture among IKEA customers in Norway and England, using the conjoint analysis (CA) and contingent valuation (CV) methods. The median WTP for the

English customers were estimated at 16.4% and 7.5% based on the CA and CV methods, respectively. The Norwegian customers have a much lower WTP of 2% and 4%, respectively.

It is, however, reported that there is little or no local demand for CTPs in developing producer countries (Durst *et al*., 2006; Espach, 2006; Miyata, 2007). Consumers in the Asian countries are said to have little interest in CTPs (Gale, 2006). The probability of gaining any price premium for CTPs is also said to be poor as consumers in the developing countries can not afford to be environmentally ethical in their consumption (van Kempen *et al*., 2009). In Malaysia, for example, there seems to be no effort to market such wood products locally, despite the fact that the country is a producer and exporter of tropical CTPs (Mohamed, 2008). Even though the more affluent and developed countries may continue to be major markets for these CTPs, there is little empirical evidence to show that such wood products have no potential in a developing country like Malaysia. This paper presents the findings of a study that investigated consumers' preference and WTP price premiums for environmentally certified wooden household furniture (ECWHF) in Malaysia. In this paper, the magnitude of the price premium the Malaysian consumers are willing to pay was also estimated.

METHOD

A mall-intercept survey using a self-administered questionnaire was conducted in 2008 to obtain the data for the study. A total of 1,048 questionnaires were distributed to systematically selected adults at four shopping malls in Kuala Lumpur, Malaysia. The location of the malls were chosen to ensure that a broad cross-section of consumers were included in the study. These consumers were selected based on the previously determined criterion that every tenth adult who passed the research assistants were approached and asked to participate in the survey. The questionnaire was distributed to those who had given their consent and then collected upon completion during the survey.

In the questionnaire, the respondents were shown two pictures of identical wooden dining furniture sets. They were first asked to decide as to which set they would choose in a hypothetical wooden dining furniture purchase situation. It was indicated to the respondents that the only difference between the two furniture sets was the type of the timber used to make the items (certified versus non-certified timbers), while price, design, quality and other attributes are identical. To ensure that the respondents understood the meaning of certified timbers, the following definition was included in each questionnaire: "*Forest certification is a system of forest inspection plus a means of tracking timber through a "chain of custody" – following the raw material through to the finished product. The goal of forest certification is to ensure that the products have come from forests which are well managed – meaning its management takes into account environmental, social and economic benefits of the forests.*

Timbers which come from forests which are certified are thus certified timbers". This definition was repeated three times in the questionnaire. The respondents were given the following response options: "Choose set made from certified timbers", "Choose set made from non-certified timbers", "Would choose either set", and "Don't know". Nonetheless, the respondents were not asked about WTP.

The respondents answering "Choose set made from non-certified timbers" were asked which of the several statements best described the reason for not choosing the dining furniture set made from certified timbers, whereas those indicating a preference for the dining set made from certified timbers ("Choose set made from certified timbers" response), an indifference ("Would choose either set" response) or uncertainty ("Don't know" response) were asked about their WTP. A contingent valuation method (CVM), with single-bounded dichotomous choice questioning format regarding WTP a price premium for CTPs, was used in this study. The method is currently the standard approach used to elicit consumers' WTP, which can be conducted by direct survey via telephone, mail or face-to-face (Li *et al*., 2002). In the dichotomous choice CVM, each respondent was asked for his/her WTP a particular price for a particular good in a hypothetical market with a "YES" or "NO" option to the premium offered (McCluskey *et al*., 2005).

The stated preference scenario given to respondents was: "*You may have to pay a higher price for wood products made from certified timbers due to the costs of getting certified, maintaining certification, and segregation in the production and marketing systems. Would you be willing to pay if it costs more to buy a set which is made from certified timbers than the set which is made from non-certified timbers?*" Those who answered this question in the affirmative were then asked "Would you be willing to pay an extra RMXXX for the set made from certified timbers?" The hypothetical initial bid price for both furniture sets was RM2000 and the premium for the wooden furniture set made from certified timbers was offered at one of the following bid price levels: RM100, RM200, RM300, RM400 and RM500. The premium amounts were selected based on an earlier study conducted by Mohamed and Ibrahim (2007). Each respondent faced only one randomly assigned premium. The respondents who answered negatively were asked which of the several statements best described the reason for not willing to pay a premium.

RESULTS AND DISCUSSION

General Characteristics of the Sample

After eliminating incomplete and erroneous questionnaires, only 994 questionnaires were used in the analysis. The majority of the respondents were Malays (74.4%) and slightly more than half were females (52.2%). The average monthly income of the respondents was about RM2372 (RM3.08 to USD1) and their average age was 32 years. The average education level of the respondents was equivalent to a certificate, which is usually a two-year post-

secondary school formal education. About 76.7% of the respondents currently own a wooden dining furniture set at home. A summary of the respondents' demographics is shown in Table 1 below.

TABLE 1
Respondents' demographic information

Characteristics	Percentage (%)
Gender	
Male	47.79
Female	52.21
Age	
30 years and below	53.82
31 – 40 years	27.16
41 – 50 years	13.78
51 – 60 years	4.73
61 years and above	0.51
Ethnic	
Malay	74.44
Chinese	14.89
Indian and others	10.67
Education	
At least 6 years (primary)	3.82
At least 13 years (secondary)	30.08
At least 15 years (certificate)	13.88
At least 16 years (diploma)	23.64
At least 17 years (university degree)	28.57
Monthly gross income	
RM2000 and below	58.15
RM2001 – 4000	30.88
RM4001 – 6000	5.53
RM6001 and above	3.32
(Missing cases: 51)	

Preference for Wooden Dining Furniture Set Made from Certified Timbers

A majority (74.0%) of the respondents showed a preference for the wooden dining furniture set made from certified timbers when asked to make a choice in the hypothetical wooden furniture purchase situation given in the survey (Table 2). Some studies have reported similar consumer's/ customers' propensity to choose CTPs over its identical non-certified products, especially when both items are priced at the same level. For example, about 94.3% of the customers in British Columbia's home improvement market interviewed by Forsyth *et al*. (1999) indicated that they would choose a certified wood product if it was priced at the same level as its non-certified competitor. An experiment conducted by Anderson and Hansen (2004) at two Home Depot outlets in Oregon, USA also showed that a large percentage of the consumers preferred to buy certified plywood when offered at a similar price over the identical uncertified product.

TABLE 2
Distribution of the respondents' responses to hypothetical furniture purchase situation

Respondents' choice	Frequency	Percentage (%)
Choose set made from certified timbers (Preference)	736	74.0
Would choose either set (Indifferent)	150	15.1
Don't know (Uncertain)	75	7.5
Choose set made from non-certified timbers	33	3.4
Total	994	100.0

About 15.1% and 7.5% of the respondents are either indifferent or uncertain of their choice, respectively.

Meanwhile, the remaining percentage (3.4%) of the respondents chose the wooden dining furniture set made from non-certified timbers. The commonly mentioned reasons for their choice were that they believe both types of timber are similar and that certified timbers are not necessary as the forests in the country should have been well-managed.

Incidence of Consumers' WTP Price Premiums

The WTP component of the study involved determining whether the respondents would be willing to pay a price premium for the wooden dining furniture set made from certified timbers and their WTP one of the five bid price premiums offered. Only about 61.5% of those who had indicated their preference for the wooden furniture set made from certified timbers were found to be willing to pay a price premium for the product (Table 3). Much lower percentages (53.3% and 38.7%) of those who were indifferent and uncertain about their choices were shown to be willing to pay more for the set, respectively.

TABLE 3
Respondents' willingness to pay price premiums for wooden furniture set made from certified timbers

Respondent's choice of wooden furniture set made from certified timbers	WTP price premium		
Total	Yes	No	
Preference	453	283	736
Indifferent	80	70	150
Uncertain	29	46	75
Total	562	399	961

Note: Thirty-three respondents chose the wooden furniture set made from non-certified timbers

TABLE 4
Distribution of the responses by premium amount

WTP response	Premium offered (RM)					
	100	200	300	400	500	Total
Yes	121	77	76	76	55	405
No	13	12	37	47	48	157
Total	134	89	113	123	103	562

Upon further elicitation, not all of the 562 respondents who had indicated their WTP a price premium responded affirmatively to the premium offered to them. In particular, only 72.1% of these respondents were willing to pay a premium for the wooden furniture set made from certified timbers. The distribution of the responses for the various premium levels is shown in Table 4. It is worth noting that the percentage of the respondents indicating a positive WTP decreased with an increase in the premium offered. For example, 90.3% of those offered a premium of RM100 indicated a positive WTP, whereas only 53.4% were willing to pay a RM500 price premium for the wooden dining furniture set made from certified timbers. Other studies (e.g., Ozanne & Vlosky, 1997; Forsyth *et al*., 1999; Anderson *et al*., 2005) also reported a similar inverse relationship between WTP and the amount of premium offered. The remaining 27.9% mentioned reasons like they could not afford to pay more, they did not believe it would cost more to make wood products from certified timbers, or that manufacturers should not charge higher prices even when it costs more to make wood products from certified timbers for not willing to pay the price premium offered to them.

Estimate of Consumers' Mean WTP

Parametric or non-parametric approaches can be used to estimate the mean WTP from dichotomous choice contingent valuation questions. The respondents' mean WTP for the wooden dining furniture set made from certified timbers was calculated using the Turnbull lower-bound nonparametric estimator. The estimator is a good alternative to other parametric estimates if only the mean WTP is to be estimated (Loureiro *et al*., 2009). The calculation following that of Ahtiainen (2007) is shown in Table 5. The results showed that the respondents, on average, were willing to pay an additional RM359.27 for the wooden dining furniture set made from certified timbers. This represents a premium of almost 18% over the set made from non-certified timbers.

TABLE 5
Turnbull estimate of the lower bound on the sample mean

Lower bound of interval	Upper bound of interval	Probability of answering *yes* at upper bound	Change in density
RM0	RM100	0.9030	0.0970
RM100	RM200	0.8652	0.0378
RM200	RM300	0.6726	0.1926
RM300	RM400	0.6179	0.0547
RM400	RM500	0.5340	0.0839
RM500	∞	0	0.5340

Estimate of lower bound mean:
RM0 * 0.0970 + RM100 * 0.0378 + RM200 * 0.1926 + RM300 * 0.0547 + RM400 * 0.0839 + RM500 * 0.5340 = RM359.27

CONCLUSION

The results of this study have shown that there is a consumer preference for CTPs in Malaysia. About 74% of the respondents in the study had expressed their willingness to buy wooden household furniture made from certified timbers if they were priced at similar level with identical non-certified products. Meanwhile, other 15.1% would probably choose ECWHF in a similar purchase situation. However, the number of consumers who will choose CTPs is expected to decline when they have to pay price premiums for them. This is consistent with the findings of other research, whereby the number of those expressing a positive WTP decreases with an increase in the amount of premium. Overall, only 40.7% of the consumers were found to be willing to pay a price premium for CTPs. On average, consumers in Malaysia were willing to pay about 18% more for CTPs over their identical non-certified competitor.

Thus, it is important to note that while there appear to be a preference and WTP a premium for CTPs among consumers in Malaysia, a discrepancy between the actual consumers' behaviour and their stated intention may occur. This is because consumers' purchase of wood products, in this case wooden household furniture, would also be influenced by other product attributes like quality, design, functionality and price. However, the results have shown that there is a potential for CTPs in a developing country like Malaysia. CTPs may be appropriate for specific niche markets, which should be developed by marketers of these wood products. Hence, identification of the characteristics of the consumers, who will make up the niche markets, should be attempted.

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Anatomical Structures of the Limb of White-nest Swiftlet (*Aerodramus fuciphagus*) and White-headed Munia (*Lonchura maja*)

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ABSTRACT

The main aims of this study were to examine the anatomical structures of the pelvic limb of white-nest swiftlet and to find the reason why the birds are not able to walk, stand and perch while standing. The findings were compared with the white-headed munia which has almost similar body weight and appearance, and the above-mentioned abilities. Four left limbs from each type of the birds were examined macroscopically under the stereomicroscope, whereas the bones and muscles of both the species were measured and compared. The lengths of the femur and tibial bones of the two species were not significantly different, although the metatarsal bone and digits of the white-nest swiftlet were found to be shorter than those of the white-headed munia. In particular, the digits of the white-nest swiftlet were shorter and curvy as compared to the white-headed munia which has longer digits with straight and sharp claws. The limb muscles of white-nest swiftlets were smaller and thinner than the white-headed munia. Four muscle groups, namely, bicep femoris, semimembranous, semitendinosus and gastrocnemius, were also taken from each bird for histological examination. The muscle sections were stained with Haematoxylin and Eosin. Histologically, the white-nest swiftlets have relatively smaller muscle groups and muscle bundles as compared to the white-headed munia. Thus, the limb is weak and unable to support its body weight. In conclusion, apart from the short metatarsal bone and digits, the small muscles of the limb could be the main reason for the inability of the white-nest swiftlets to use their limbs for walking, standing and perching while standing.

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INTRODUCTION

White-nest swiftlets (*Aerodramus fuchipagus*) belong to the genus *Aerodramus* of small, dark, cave nesting birds in the Collocaliini tribe of the swift family *Apodidae*. Its members are confined to tropical and subtropical regions in Southern Asia, Oceonia and North-eastern Australia. Many of its members were formerly classified in Collocalia, but were first placed in a separate genus by an American ornithologist, Harry Church, in 1906. Echolocation, DNA sequencing and parasitic lice have all been used to establish relationships between species (Chantler & Driessens, 2000). *Aerodramus* swiftlets are in many respects typical swifts, having narrow wings for fast flight, with a wide gap and small reduced beak surrounded by bristles for catching insects during flight. What distinguishes *Aerodramus fuciphagus* from other swifts, and indeed from almost all other birds, is their ability to use a simple but effective form of echolocation. This enables them to navigate within the breeding and roosting caves. The swiftlet's "sonar" consists of clicking sounds at the frequencies of 1,500 to 5,500 hertz, which are audible to the human ear and are emitted at the rate of about six times per second (Gausset, 2004). The nests of *Aerodramus fuciphagus* are constructed with saliva as a major component. In the two species, *Aerodramus fuciphagus* and the Black-nest Swiftlet or *Aerodramus maximus*, saliva is the only material used, and the nests are collected for the famous Chinese delicacy known as "bird's nest soup". Nonetheless, over-collection has put pressure on the swiftlet populations (Jordan, 2004).

The white-headed munia (*Lonchura maja*) is a species of estrildid finch found in Indonesia, Malaysia, Singapore, Thailand and Vietnam. It is found in wetland habitat, especially in marshes and reeds (Crystal, 2010). Generally, they are similar to black-headed or chestnut munia, but have paler brown to whitish on the entire head and the throat is white. Young birds are brown on the upper parts with under parts and the face is buff. In Java and Bali, this is a fairly common and widespread bird in the area up to 1500 metres in height. The white-headed munia, like other munias, form large flocks during rice harvest but spread out in pairs during breeding season. The general behaviour of this species is similar to other munias (Crystal, 2010).

In this study, the pelvic limb muscles and bones of both species were grossly and histologically examined for the cross-sectional area of each muscle and muscle bundles of the thigh. The muscles taken for histology were only the prominent and important muscles for movement. To the authors' knowledge, the limb of white-nest swiftlets is not able to support their body weight, thus preventing the birds from standing and perching while standing, but allowing them to cling onto the vertical surface. However, the anatomical structure of the limb of swiftlets has not been fully documented. Thus, this study

was conducted with the objective to examine the differences in anatomical structures of the pelvic limb of the white-nest swiftlets (*Aerodramus fuciphagus*) and the white-headed munia (*Lonchura maja*).

MATERIALS AND METHODS

Birds and Sample Preparation

The study involved four adult birds from each white-nest swiftlet (*Aerodramus fuciphagus*) and white-headed munia (*Lonchura maja*). The white-nest swiftlets were taken from a farm in Tersat, Terengganu, in collaboration with the Department of Veterinary Services. The whole left pelvic limb from each bird was separated for dissecting. The white-headed munias were bought from a local bird shop in Sri Serdang. The birds were euthanized by cervical dislocation. The whole pelvic limbs of the left side of both birds were taken and fixed in the 10% formalin for two days before processed for histological examinations.

Macroscopic Examinations

The macroscopic examination of the pelvic limb was done under a stereomicroscope after two days of fixation in 10% buffered formalin. The muscles of the thigh and the leg of both the species of birds were dissected, measured and recorded. The dissected muscles of the limb were photographed and compared between the two species. The bones of the pelvic limb, which include the femur, tibia, metatarsus and digits, were also measured and recorded.

Histological Examinations

From each bird, the muscles of the left pelvic limb (*M. biceps femoris*, *M. semimembranosus, M. semitendinosus and M. gatrocnemius*) were taken for histological examinations. All the samples were washed with phosphate buffered normal saline pH 7.4, fixed in 10% neutral buffer formalin for 24 hr, and processed using standard histological procedures. Sections of 5µm thick were cut using a microtome (Leica 2045). The sections were mounted onto the glass slides and stained with the Haematoxylin and Eosin (Bancroft & Gamble, 2005). The sections were evaluated using a computerized image analyzer (Olympus image analysis, BX 51 TF) that was equipped with a camera CC12. The measurements of the muscle cross-sectional area and muscle bundle were performed by using a light microscope (Leica DM LB2, Germany) using a colour video camera. For each sample, six bundles were randomly and constantly selected in 100x magnifications, as well as measured at the middle of the bundle. The cross-sectional areas of the muscles and muscle bundles were measured under the same magnification.

Statistical Analysis

The means for the cross-sectional area of the muscles, cross-sectional area of the muscle bundles, the length of the femur, tibia and metatarsus, and the digits of the white-nest swiftlets and white-headed munia were analyzed using independent T test to compare the differences between the two

species. All the statistical analyses were performed using SPSS 12.0.

RESULTS

Macroscopic Examinations

Fig.1 shows the mean lengths of the pelvic limb bones of the white-nest swiftlets and white-headed munias. The results revealed that the length of femur (11 ± .1 and 13 ± .8 for the white-nest swiftlets and white-headed munias, respectively) and tibia bones (17 ± .3 and 20 ± .6 for the white-nest swiftlets and white-headed munias, respectively) were not significantly different (P>0.05), although they were slightly shorter in white-nest swiftlets. However, the metatarsus (9 ± .4 and 14 ± .2 for the white-nest swiftlet and white-headed munia, respectively) and digits (4 ± .5 and 9 ± .4 for the white-nest swiftlet and white-headed munia, respectively) were significantly shorter (P<0.05) in the white-nest swiftlets than those of the white-headed munias (see Fig.2 and Fig.3). The digits of the white-nest swiftlets were short with curvy claws, while the digits of white-headed munia were longer, and the claws were rather straight and sharp (Fig.3).

All the pelvic limb muscles examined in this study were present in both the species. However, the size was very much different between the two species. Both the thigh and tibiotarsal muscles of the white-nest swiftlets were found to have smaller size as compared to the white-headed munias (see Fig.4 and Fig.5). Those muscles include the biceps femoris, semimembranosus, semitendinosus, quadriceps femoris, tensor faciae latae, gastrocnemius muscles, deep digital flexor muscle and long digital

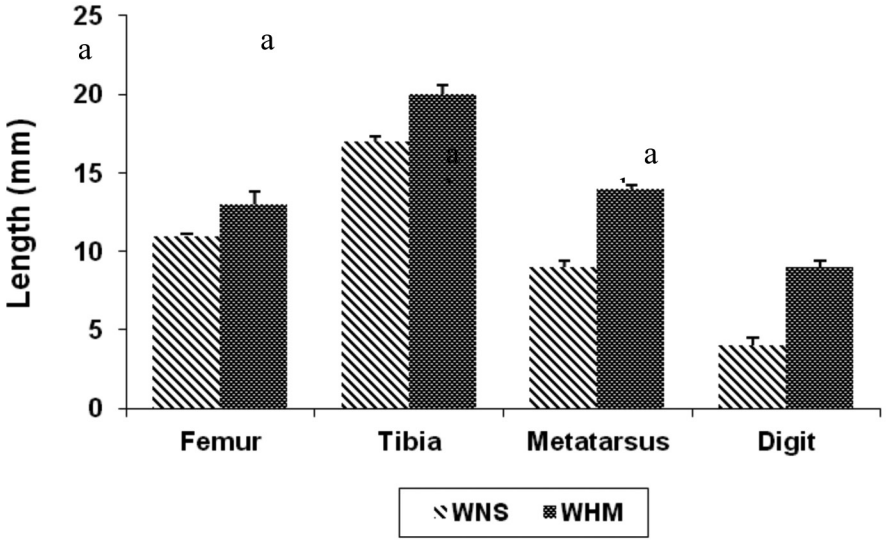

Fig.1: The white-nest swiflets' (WNS) and white-headed munias' (WHM) length of the pelvic limb bones. Values are mean ± SE. a, b different symbols indicate significant differences (P< .05)

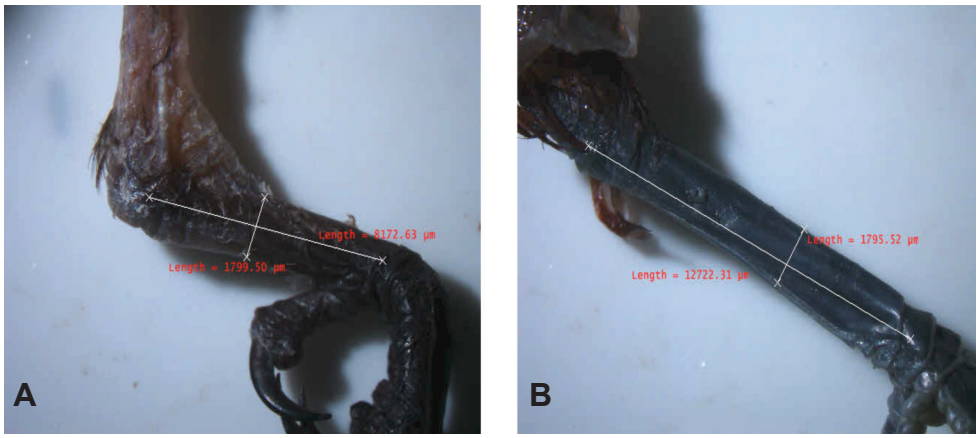

Fig.2: Photographs showing the metatarsal bone of (A) the white-nest swiftlet, and (B) white-headed munia. The metatarsal bone in A is much shorter than in B

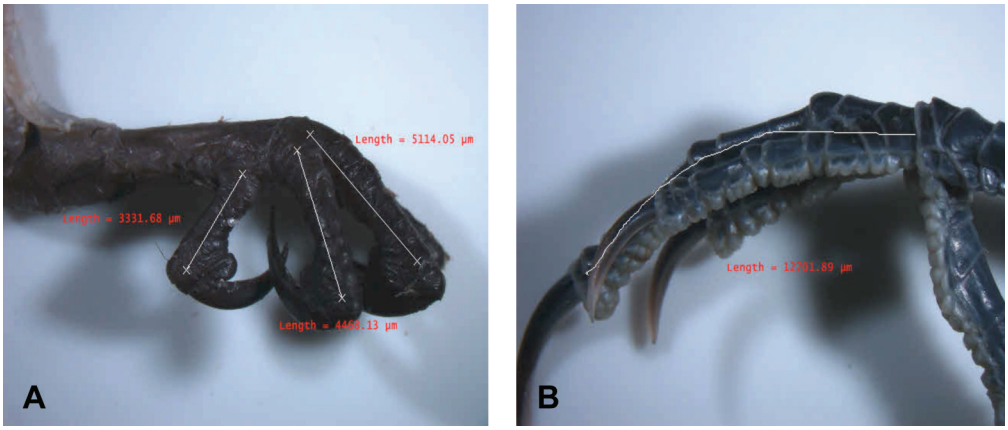

Fig.3: Photographs of the digits of (A) the white-nest swiftlet, and (B) white-headed munia. The digits of the white-nest swiftlets are much shorter and curvy claw, which are suitable for clinging or hanging, while these are longer with straight claws for the white-headed munias for standing and perching purposes

extensor muscle. Nonetheless, the fibularis longus muscles of both species were found to be similar in size. The thigh muscles of the white-nest swiftlets were small and thin, which allow the femoral bone to be seen grossly, as shown in Fig.4.

Histological Examinations

Muscle Groups Area

The cross-sectional areas of the muscle groups of the white-nest swiftlets and white-headed munias are shown in Fig.6. The area of all the four selected muscles of the white-

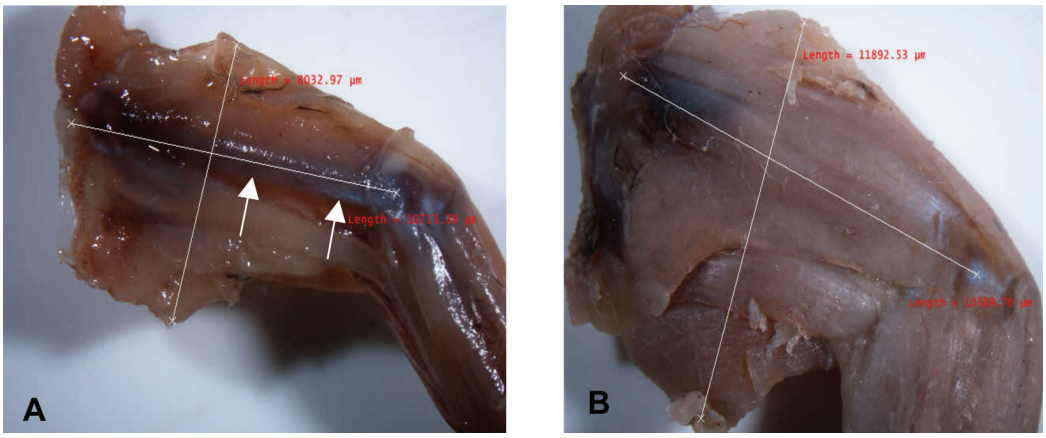

Fig.4: Photographs showing the thigh muscles of (A) the white-nest swiftlets and (B) white-headed munias. The thigh muscles in (A) are much smaller than in (B). Also note that the femur is grossly visible in (A) (arrows)

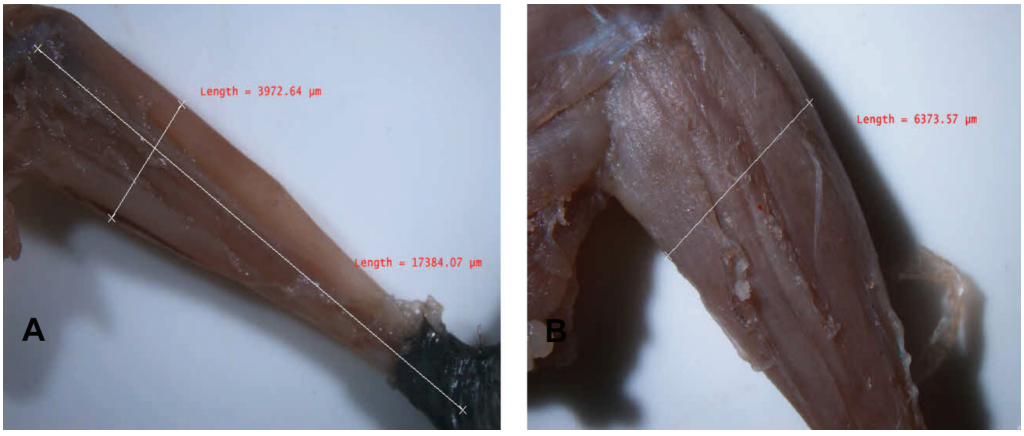

Fig.5: Photographs showing the muscles of the tibiotarsus of (A) the white-nest swiftlets and (B) white-headed munias. The tibiotarsal muscles in (A) are much smaller than in (B)

nest swiftlets were significantly smaller ($P<0.05$) than the white-headed munia. In addition, the semitendinosus muscle of the white-nest swiftlets was particularly almost negligible.

Muscle Bundles Areas

The cross-sectional areas of the muscle bundles of the white-headed munias and white-nest swiftlets are shown in Fig.7. The areas of the muscle bundles for the three muscle groups, namely the biceps femoris, gastrocnemius and semimembranosus muscles, were found to be significantly smaller ($P<0.05$) in the white-nest swiflets. In addition, the semimembranosus muscle bundles of the white-nest swiftlet were found to be smaller ($P<0.05$) than the white-headed munias.

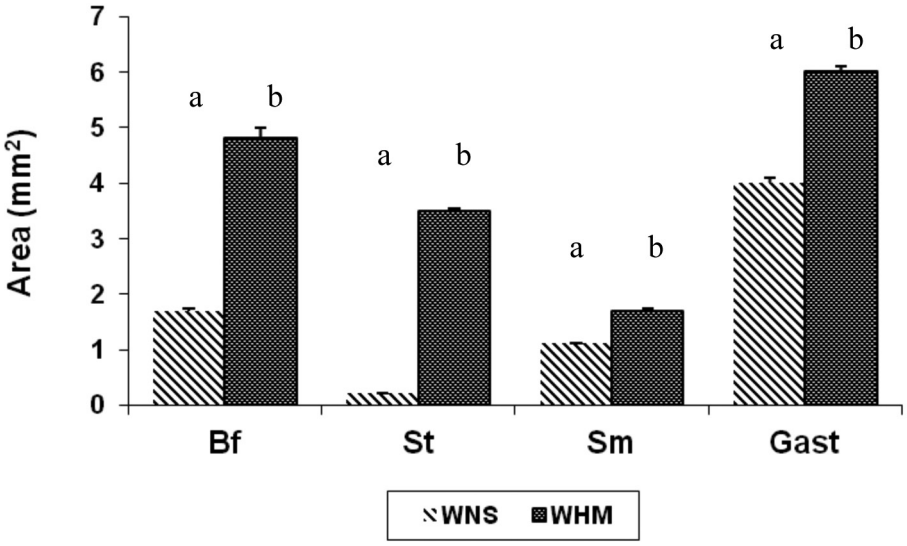

Fig.6: The cross-sectional area of the muscle groups of the white-headed munias and white-nest swiftlets; biceps femoris (Bf); semitendinosus(St); semimembranosus(Sm); gastrocnemius (Gast). The values are mean ± SE a,b different symbols indicating significant differences (P< .05)

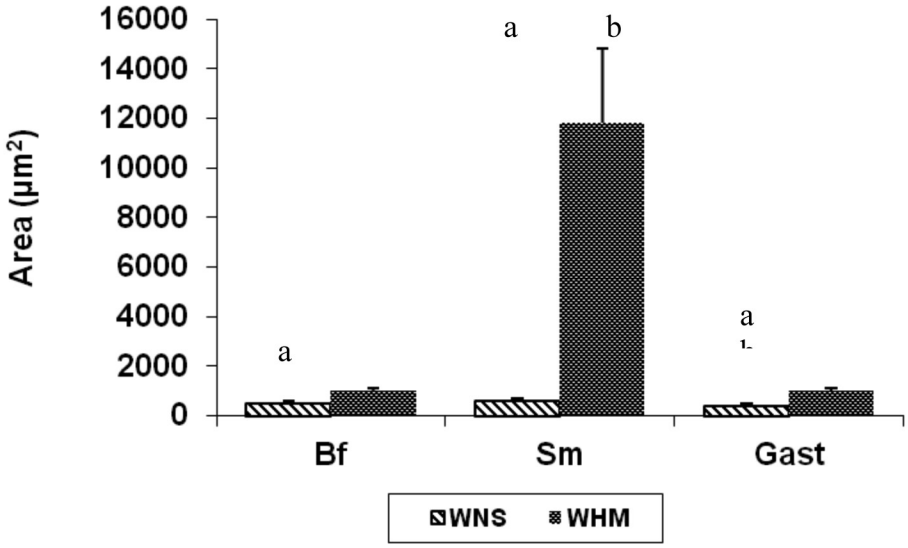

Fig.7: The cross-sectional area of the muscle bundles of the white-nest swiftlets (WNS) and white-headed munias (WHM). Biceps femoris (Bf); semimembranosus (Sm); gastrocnemius muscle (Gast). The values are means ± SE. a,b different symbols indicating significant differences (P<.05)

DISCUSSION

Studies on the pelvic myology of the non-passerine birds are numerous. Among those which appear to be the most notable include the studies of the comparative functional morphology of the pelvic appendage in three genera of Cuculidae (Berger, 1952), the pelvic musculature in galliform birds (Hudson *et al*., 1959), the anatomy of the locomotor apparatus of New World vultures (Fisher,1946), the pelvic appendages of the Falconiformes (Hudson, 1937), the perforated flexor muscles in birds (Mitchell, 1894), the muscles of the avian (chiefly galliform) hip and thigh (Howell, 1938), and the peroneal muscles of various species (Mitchell, 1913).

The perching birds have the same requirements for the flexion and extension of the toes, a function that is performed by the muscles of the tibiotarsus and tarsometatarsus. The femur possesses muscles that are used in protracting and retracting the leg, as well as moving it in a lateral direction. These activities may be altered by variations in thigh musculature to compensate for the environmental requirements without affecting the success of perching. Therefore, the thigh is the region in which the greatest muscular variation may be expected.

In this study, the white-nest swiftlet *(Aerodramus fuciphagus)* from the family of Apodidae and the white-headed munia *(Lonchura maja)* in the family of *Estrildidae* were of the same body size and appearance; the difference between them is that the white-nest swiftlets lack the ability to walk on the ground and perch while standing. The visible pelvic limb muscles, which are large enough and have the main effects on the movement and standing for both the birds, have been successfully identified and recognised. The results revealed that limb muscles observed in the white-nest swiftlets were also present in the white-headed munia. Thus, the white-nest swiftlets cannot be categorized as incapable birds.

The thigh and tibiotarsal muscles on the white-nest swiftlets, however, were smaller and thinner than the white-headed munias. Thus, this suggested that the pelvic limb muscles of the swiftlets were less developed as compared to the white-headed munias. The thigh muscles of the white-nest swiftlets were very small and thin that they caused the femur bone to be grossly visible. In contrast, the pelvic limb muscles of the white-headed munia were bigger and well developed. Thus, the reasons why the white-nest swiftlets are unable to walk or perch while standing can be explained by the small size of the limb muscles, in addition to the short metatarsus and digits.

According to Coues (1903), the high development of the shank muscles, which flex and extend the toes, has eliminated the need for a maximum development of the toe muscles. Furthermore, most of the toe muscles are for lateral movement of the toes, a function which is not beneficial to perch. Perching birds may be expected to have better developed toe muscles than terrestrial birds because of the need to maintain a constant flexed position (Coues, 1903). The results obtained for the white-headed munia

in this study agree with those by Coues (1903). However, this study revealed that in white-nest swiftlets, the thigh muscles were undeveloped and thus the ability to perch while standing is diminished.

The findings of this study also revealed that the metatarsus of the white-nest swiftlets was shorter than that of the white-headed munias. In addition, the digits of white-nest swiftlets were also short and curvy, which are suitable for clinging or hanging, whereas, the digits of white-headed munias were longer and straight with sharp claws that play an important role in standing and perching while standing.

Hence, the histological examinations of the cross-sectional muscle areas have revealed that the muscles area of four muscles in the white-nest swiftlets are smaller than those of the white-headed munias. In addition, the semitendinosus muscle area of the white-nest swiftlet was almost negligible, with very few muscle bundles present surrounded by the connective tissues. For the white-headed munias, on the contrary, the semitendinosus cross-section area was larger. This further suggested that the pelvic limb muscles of the white-headed munias were well developed as compared to the pelvic limb muscles of the white-nest swiftlets.

Muscle bundles are important to represent the whole muscle sizes. Thus, the measurement of the muscle bundles for each bird was taken. For the white-nest swiftlets, the sizes of the three muscle bundles (namely, biceps femoris, semimembranosus and gastrocnemius) were smaller than those of the white-headed munias. The muscle bundles of the semimembranosus of the white-nest swiftlets were almost unrecognisable due to the presence of only a few muscle bundles which are surrounded mainly by the connective tissues. For the white-headed munias, on the contrary, the muscle bundles of the semimembranosus were much bigger and well-developed.

The results of this study agree with those of Hudson (1937) who noted that the eight muscles and the vinculum between Mm. flexor *perforans et perforatus digiti* III and flexor *perforatus digiti* III were missing in the order *Passeriformes*. In this study, all the eight muscles and the vinculum were missing in the white-headed munias (order *Passeriformes*) and the white-nest swiftlets (order *Apodiformes*). In addition, *Mm. adductor digiti* IV and lumbricalis were also absent. The muscles that were absent in the white-headed munias and the white-nest swiftlets were also absent in *Tyrannidae* (Tommy, 1971), Redwinged Blackbird (*Agelaius phoenicus*) (Berger & George, 1966) and House Sparrow (*Passer domesticus*) (Berlin, 1963).

Hudson (1937) described the presence of the muscles that could be shown only by special staining techniques. Since the muscle structures are rudimentary and without tendons of insertion, their importance is questionable. Illustrating the presence of the muscles using the staining techniques, nonetheless, could produce misleading muscle formulae. The natural behaviours of the white-nest swiftlets (which are always on the air most of their

time, and using the wings rather than limbs) have weaken the muscles due to undeveloped pelvic limb muscles.

In conclusion, the lengths of the femur and tibial bones in both the species of birds were not significantly different, although the metatarsal bone of the white-nest swiftlets was shorter than that of the white-headed munias. The most significant findings in this study were the smaller and thinner muscles of the pelvic limb of the white-nest swiftlets as compared to the white-headed munias. Thus, the findings suggest that the limb muscles of the white-nest swiftlets are undeveloped, and this has caused them to become are weak and unable to support their own body weight.

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Three Months' Monitoring of Environmental Factors, Biomass, Length and Size Classes Variation of *Sargassum* Species at Cape Rachado, Port Dickson

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ABSTRACT

Seasonality in biomass, thallus length and size classes of three *Sargassum* species, namely, *S. baccularia (Mertens) C. Agardh, S. binderi Sonder ex J. Agardh* and *S. siliquosum J. Agardh*, was analysed based on destructive sampling using line-transect-quadrat method from October to December 2008. Results showed that *S. baccularia* was most abundant among the three species. The plant was frequently found in the length class of 0 – 4.9 cm (79.68 %), and this was followed by *S. binderi* in length class of 5.0 – 9.9 cm (44.12 %), and *S. siliquosum* in the length class of 0 – 4.9 cm (66.67 %). The *Sargassum* species were observed to increase gradually in their biomass and mean thallus length further away from shore. Within three months, *S. baccularia* experienced a growth in its biomass and mean thallus length, while both *S. binderi* and *S. siliquosum* experienced a decrease in terms of biomass but an increase in their mean thallus length. Data also showed a correlation with environmental parameters, such as pH, DO, salinity, nitrate, phosphate and ammonia.

Keywords: Biomass, Cape Rachado, Port Dickson, Environmental parameters, Mean thallus length, *Sargassum*

INTRODUCTION

Seaweeds are macroscopic algae that can be divided into *Chlorophyceae, Rhodophyceae* and *Phaeophyceae*, based on their colour pigment. Lüning (1990) stated that diversity of seaweed species worldwide includes a rough figure between 6500 to 8000 species. Under *Phaeophyta,* more than 400 species have been estimated as belonging to the genus of *Sargassum* (Wong & Phang, 2004).

Seaweeds have enormous potential to be used as raw materials in producing many economically important products. Besides

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being eaten and used as fertilizer, seaweeds contain many commercially important polysaccharides such as agar, alginate and fucoids. Seaweeds, especially those of the *Sargassum* species, have also been utilized in the bioremediation of contaminated water (Bina *et al*., 2006).

The site of the study, i.e. Cape Rachado, is a stretch of coast surrounded by coral reefs, sandy beaches, rocky shores and mangroves. Each of these geographical areas exposes seaweeds to different environmental stresses that allow the growth of only a few selected species. This is evident in the zonation patterns caused by gradually varying parameters from shore towards sea. Ooi (2001) pointed out that diversity and abundance of seaweeds in a particular area could also give a rough indication as to the general health of a particular ecosystem.

Seaweeds show a seasonal growth cycle that is caused by climatic changes occurring throughout the year. In particular, *Sargassum* has been shown to exhibit seasonal cycles of growth, reproduction, senescence and die back (Ang, 2006). Thus, phenological studies on these seaweeds are important to provide precious information for local seaweed cultivation. The objectives of this study were to determine the diversity and the abundance of *Sargassum* species found along the fringing coral reef flats of Cape Rachado, Port Dickson, compare the zonation patterns of *Sargassum* species and also determine the growth of *Sargassum* species over a period of three months in relation to the varying environmental parameters.

MATERIALS AND METHODS

Samples of seaweed were collected from Cape Rachado on a monthly basis from October to December 2008. Line-transect and systematic quadrat sampling methods were employed. Three 100 m line-transects, marked as Line 1, Line 2 and Line 3, were placed perpendicular to the shore. On each line, a 0.09 m^2 (0.3 m × 0.3 m) quadrat was placed every 10 m interval and all seaweeds within each quadrat were harvested and placed separately in labelled plastic bags. Water sampling data were taken at the site using portable HANNA meter (HI 98280, USA). In addition, seawater samples were collected for nutrient analysis.

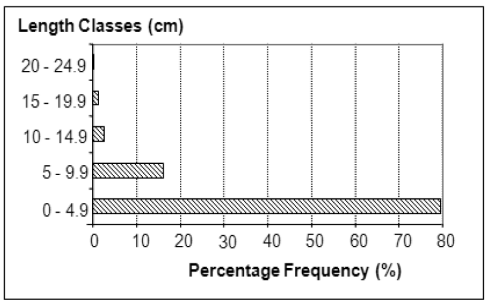
In the laboratory, water samples were tested for ammonia, nitrate and phosphate concentrations using Hach meter (DR/890, USA) while pH was measured using a pH meter (Delta 320, China). The seaweed samples were washed thoroughly with tap water, after which, three different species of *Sargassum* were identified and separated according to their quadrats. Individual samples were also measured for their lengths and weighed according to species per quadrat using an analytical balance (Adventurer™ Pro Av812, USA) to obtain the wet weight per quadrat. As for the dry weight per quadrat, the samples were oven-dried at 105 °C for 48 hours and then reweighed. Biomass per quadrat was divided by the area of the quadrat (0.09 m^2) and recorded in g WW m^{-2} for wet weight and g DW m^{-2} for dry weight. Thallus length of all the plants was measured before obtaining wet weight. The thallus length

was measured as the distance from the end of the holdfast to the apex of the longest branch. The measured length of all the plants was averaged to give the mean plant length. The overall percentage coverage of each species in the study area was obtained by dividing the number of plants from each *Sargassum* species with the total number of the *Sargassum* plants and then multiplied by 100%.

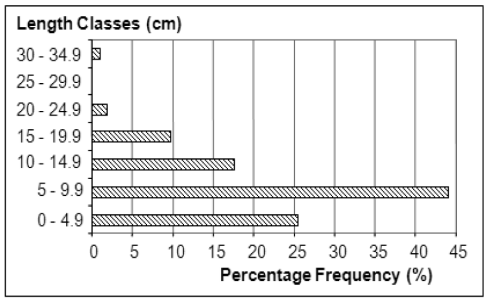
All the statistical analyses were conducted using the SPSS 15.0 software. One way ANOVA and Post Hoc Test (Tukey HSD) were also applied to determine any significant differences in biomass and mean thallus length of each *Sargassum* species between months. Meanwhile, the Pearson's correlation coefficient analysis was applied to correlate changes in the dry weight of *Sargassum* species with the environmental parameters.

RESULTS AND DISCUSSION

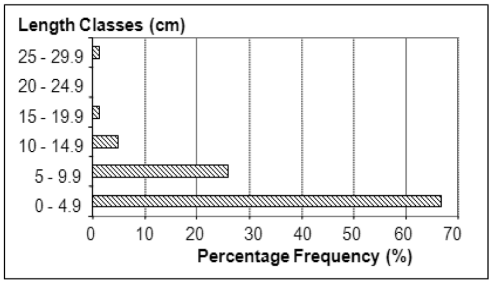
The seaweed samples found from the study site included *S. baccularia, S. binderi* and *S. siliquosum,* among a vast variety of other species. Results presented in Fig.1 show that the thallus length of *S. baccularia* most frequently range in the length class of 0.0 – 4.9 cm (79.68 %), with a maximum length of 20.0 – 24.9 cm (0.32 %). As for *S. binderi*, the samples of length class of 5.0 – 9.9 cm were most frequently found (44.12 %), with the maximum thallus length of 30.0 – 34.9



(A) *S. baccularia*



(B) *S. binderi*



(C) *S. siliquosum*

Fig.1: Overall Percentage Frequency for Various Length Classes.

TABLE 1
Number of Plants and Percentage Coverage of the *Sargassum* species at Cape Rachado, Port Dickson from October to December 2008

Species	October 2008		November 2008		December 2008		Total	
	No. of Plants	%	No. of Plants	%	No. of Plants	%		%
S. baccularia	157	59.02	203	84.94	265	86.88	625	77.16
S. binderi	56	21.05	32	13.39	16	5.25	104	12.84
S. siliquosum	53	19.93	4	1.67	24	7.87	81	10.00
Total	266	100	239	100	305	100	810	100

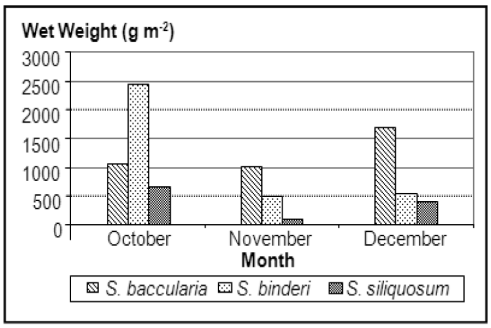
cm (0.98 %). Lastly, *S. siliquosum* was most frequently found in the length class of 0.0 – 4.9 cm (66.67 %), with the longest samples from the length class of 25.0 – 29.9 cm (1.23 %).

These obtained length classes are relatively very short compared to those found in the neighbouring countries such as Philippines (Trono, 1998) and Thailand (Noiraksa *et al*., 2006). Wong and Phang (2004) stated that *S. baccularia* and *S. binderi* plants of Cape Rachado were generally found to be in smaller length classes throughout the year. This is due to the spatial distribution that places these seaweeds at the mid to upper intertidal zone, as opposed to the species of other countries that were placed at the lower intertidal zone. Therefore, desiccation stress that is experienced by seaweeds exposed to air restricts the growth of seaweeds in Cape Rachado. Moreover, some plants were observed to detach from the holdfast once the tide comes in.

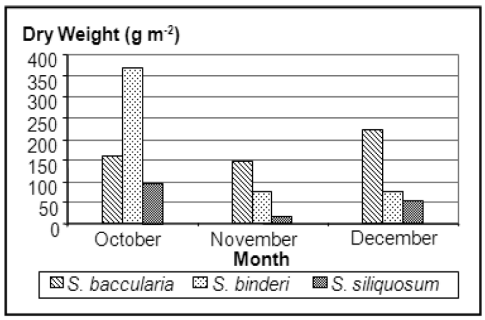
Table 1 shows that within three months, *S. baccularia* represented the most abundant *Sargassum* species along the fringing coral reef flats (77.16 %), followed by *S. binderi* (12.84 %) and *S. siliquosum* (10 %). In addition, the total number of plants collected decreased from October (266 plants) to November (239 plants), but increased in December (305 plants).

S. baccularia biomass (wet and dry weight) increased within three months, while *S. binderi* and *S. siliquosum* decreased (Fig.2). Similarly, the mean thallus lengths of *S. baccularia* and *S. binderi* decreased from October to November, but these increased in December. However, *S. siliquosum* gradually increased in length within three months. Meanwhile, the mean thallus length of *S. baccularia* was significantly different ($F = 5.707$, $p < 0.05$) between the months of November (i.e. mean thallus length = 3.43 ± 2.34 cm) and December (i.e. mean thallus length = 4.33 ± 3.05 cm) 2008; as for *S. siliquosum,* $F = 7.513$, $p < 0.05$ between October (mean thallus length = 3.49 ± 2.24 cm) and December (mean thallus length = 6.78 ± 5.73 cm) 2008.

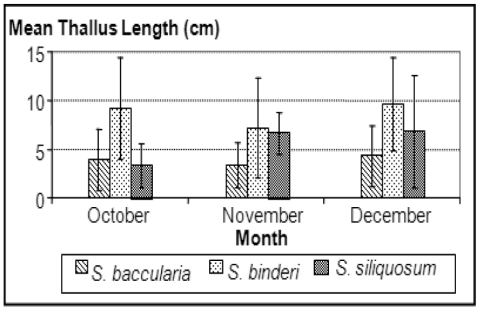
Studies by Phang (1995) and Wong (1997) reported that the abundance of *Sargassum* species peaked during the hot and dry inter-monsoon seasons but



(A) Wet Weight (g WW m^{-2})



(B) Dry Weight (g DW m^{-2})



(C) Mean Thallus Length (cm) (± SD)

Fig.2: Three months monitoring for all the *Sargassum* species.

degenerated during the wet and rainy monsoon seasons, as experienced during the period of this study. Wong (1997) observed that the peak growth and reproduction of the *Sargassum* species occurred in June 1995, but thereafter, the mean thallus length decreased up to December 1995. This represented the degeneration of seaweeds after the peak reproduction (Wong & Phang, 2004). The appearances of new recruits were evident only after a few months from the reproduction phase. This resulted in a shift of seaweed mean thallus length to smaller length classes the subsequent months, as seen in this current study. In seaweed cultivation, it would be a bad period to harvest the crops during the last quarter of the year, as opposed to middle of the year where growth is at its peak.

In addition, the changes in the environment play an important role in determining the growth or degeneration of seaweed. Table 2 records the monthly average measurements of environmental parameters. In the present study, the dry weight of the *Sargassum* species was found to correlate with environmental parameters (Table 3). *Sargassum baccularia* experience positive growth, and also increase in pH and phosphate levels. For *S. binderi* to grow, there should be increases in pH, DO and salinity, but decreases in nitrate, ammonia

TABLE 2
Averaged Measurements of Monthly Environmental Parameters

Parameters	October	November	December
*Water Temperature (°C)	29.73	-	30.2
*DO (ppm)	4.11	-	1.66
*Salinity (ppt)	29.87	-	28.79
pH	7.86	7.65	6.43
Phosphate (mg L^{-1})	0.07	0.09	0.04
Nitrate (mg L^{-1})	0.3	1.25	0.5
Ammonium (mg L^{-1})	0.02	0.05	0.04

*Measurements for November 2008 are unavailable due to faulty equipment.

TABLE 3
Correlation of dry weight (g DW m^{-2}) with environmental parameters

Parameters	Correlation Coefficient (r) for *Sargassum* species		
	S. baccularia	*S. binderi*	*S. siliquosum*
pH	0.639*	0.499*	0.363
Phosphate (mg L^{-1})	0.824*	0.386	0.925*
Nitrate (mg L^{-1})	-0.441	-0.698*	-0.532*
Ammonium (mg L^{-1})	-0.102	-0.646*	0.082
Water Temp (°C)	-0.175	-0.782*	0.189
DO (ppm)	0.427	0.918*	0.075
Salinity (ppt)	-0.061	0.613*	-0.414

* Significantly correlated ($p < 0.05$)

and water temperature. Meanwhile, for *S. siliquosum* to grow, there should be an increase in phosphate, while a decrease in the nitrate level.

It is crucial to highlight that pH, phosphate and nitrate concentrations play very important roles in the growth of *Sargassum*. These are in agreement with the results by Wong and Phang (2004), whereby the increase in *S. baccularia* biomass was found to be significantly correlated with the increase in the phosphate levels, while an increase in the dry weight of *S. binderi* was significantly correlated with the decreases in the ammonia and nitrate levels.

According to Wong and Phang (2004), rainfall was the most important factor influencing the growth of *Sargassum*. Coincidently, the period of this particular study fell in the monsoon period which receives constant rainfall. Combined with strong waves and high turbidity, this will affect the parameters tested below. For instance, slightly acidic water droplets from the rain will affect pH of seawater, which in turn discourages the growth

of seaweeds during that period. Strong waves that constantly disturb the seabed will also encourage circulations of nitrate and ammonia, and thus increasing their concentrations in seawater.

Meanwhile, high nitrate concentrations affecting *S. binderi* and *S. siliquosum* more than *S. baccularia* can be explained by the spatial distribution of these plants. In particular, *S. baccularia* has been found to be more abundant nearer to shore, while *S. binderi* and *S. siliquosum* were found more in deeper waters, or further away from shore. This indicates that both *S. binderi* and *S. siliquosum* were exposed more to the high nitrate levels detrimental to their growth.

CONCLUSIONS

In conclusion, the analysis of the length and size classes revealed that the three *Sargassum* populations comprised mainly small plants, indicating the recruitments of new plants during the three months monitoring. The important parameters affecting the biomass of *S. baccularia* are pH and phosphate, and these include all the parameters tested except for phosphate for S. binderi, whereas for *S. siliquosum* are phosphate and nitrate, respectively.

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Improvement of Malaysian Ornamental Plants through Induced Mutation

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ABSTRACT

Malaysian Nuclear Agency (Nuclear Malaysia) has started research on the improvement of ornamental plants through induced mutation (mutagenesis) since the early 1990s. The research emphasis was initially on creating new ornamental varieties through the use of the nuclear technology and later through a combination with biotechnology. Concurrently, several other species of landscaping plants, flowering and foliage were also subjected to radiation for further improvement. To date, Nuclear Malaysia has produced more than 20 new varieties of ornamental and landscaping plants. These new varieties have been transferred to various end-users, private nurseries and government agencies, such as the National Landscape Department and local councils, through collaborations and partnerships. Besides diversifying local ornamental germplasms, these efforts are also in line with the government's vision to make Malaysia a "Beautiful and Advanced Garden Nation" by the year 2020.

Keywords: Ornamental plants, induced mutation, mutation breeding

INTRODUCTION

Induced mutation is an alternative and a complementary technique in plant breeding for the introduction of genetic changes and the establishment of new genetic resources. The technology, which is also known as mutation breeding, was started in the early 1930s using mainly X-rays as a source of mutagen. Since 1950s, it has been widely used, specifically in crops with low genetic variability and those that are not amenable to improvement through conventional breeding methods. The number of physical and chemical mutagens used in mutation breeding is

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numerous and continuously increases, such as gamma rays, beta rays, neutrons, electron beams, ion beams (physical), sodium azide, ethylmethanesulfonate, and colchicines (chemical) (Ahloowalia, 2001; Medina *et al*., 2004; Jain, 2007). Through induced mutation, a large number of plant varieties have been developed with improved traits such as high yield, early maturity, as well as high protein content, biotic and abiotic resistance. According to IAEA Mutant Varieties Database (http://www-mvd.iaea.org), 2,570 mutant varieties have been officially released worldwide. Of these, 625 varieties are ornamental and decorative plants, and the improved characters include compact growth, attractive variegated leaves and novel flower colour and shapes.

Malaysian Nuclear Agency (Nuclear Malaysia), which was formerly known as Malaysian Institute for Nuclear Technology Research (MINT), has been involved in plant breeding and improvement projects since 1990s. The projects, led by the agency's Agrotechnology and Biosciences Division, mainly focussed on the application of nuclear (radiation) technologies to develop new plant varieties with commercial potentials. To date, Nuclear Malaysia has produced more than 20 new varieties of ornamental and industrial crops. Among the varieties which have been officially launched are ornamental plants such as *Hibiscus rosa-sinensies* "Siti Hasmah PinkBeauty", "Siti Hasmah RedShine" and "Nori", *Cordyline terminalis* "Teguh", "Jaguh" and "Mantap", *Cordyline fruticosa* "Shuhaii", *Duranta repens* "marginata" and "variegata", orchids (*Dendrobium* "Sonia KeenaRadiant", "Sonia KeenaOval", "Sonia KeenaAhmadSobri" and "Sonia KeenaHiengDing") and *Petunia hybrida* "NK Tropicana". The new varieties of industrial crops include *Musa cavendishi* "Novaria" and groundnuts (*Arachis hypogaea* "KARISMA Sweet" and *Arachis hypogaea* "KARISMA Serene").

A number of these varieties have been successfully transferred to end-users, which include private growers, government agencies and local councils such as the National Landscape Department, Selangor State Agriculture Department, Taiping Municipal Council, and Hexagon Green Sdn. Bhd., through collaborations and partnerships. In the recent years, molecular techniques have also been incorporated into our mutation breeding programmes to facilitate mutation selection process and develop molecular markers for mutant genes and plants. This paper summarizes mutation breeding works at Nuclear Malaysia to improve selected Malaysian ornamental plants.

MATERIALS AND METHODS

Determination of the optimum dose of irradiation treatment was carried out on both *in vivo* materials (cuttings, rhizomes, bulbs) and *in vitro* (tissue-cultured) materials [protocorm-like bodies (PLBs), nodes, leaf and petal cultures]. In the *in vivo* mutagenesis approach, cuttings, rhizomes or bulbs were irradiated at the predetermined optimum doses and then propagated vegetatively until four vegetative generations (M1V4) are

reached to ensure the stability of the mutated traits. Meanwhile, the phenotypic variations were periodically observed on irradiated plants. The mutated plants were isolated and further propagated, either through conventional propagation procedures or by tissue culture (*in vitro* procedures).

In the *in vitro* mutagenesis approach, similar procedures were followed except that the irradiation treatment was done on the tissue-cultured materials. Prior to this, the most suitable medium for the micropropagation had been formulated for various species and explants. The irradiated explants were multiplied until the fourth subculture cycle (M1V4) before they were transplanted in the nursery. Observations on the morphological changes in irradiated plants were done at both *in vitro* and *in vivo* stages. Selected mutant(s) were then conventionally propagated or tissue-cultured to obtain clonal mutant lines.

RESULTS AND DISCUSSION

Mutation breeding programme for the ornamental plants at Nuclear Malaysia was initially aimed at improving plant and flower characteristics, which were very difficult to achieve through conventional breeding. Characters of interest for improvement were new flower/leaf colours and morphology using acute gamma radiation. The first batch of ornamental mutant varieties was officially launched in 2000 by Tun Dr. Siti Hasmah Mohd Ali, during Nuclear Malaysia Flora Day. Since then, newly developed mutants, either of ornamental or food crops, were launched almost every year during the same or similar events. Details on the characteristics of the new ornamental mutants are shown in Table 1. In addition to ornamental plants, Nuclear Malaysia has also released mutant varieties of food and industrial crops through gamma irradiation, such as bananas, in collaboration with International Atomic Energy Agency or IAEA (1994), groundnut (2002) and signal grass, in collaboration with Veterinary Institute, Kluang, Johor, Malaysia (2003).

Since 2003, through bilateral agreement with Japanese Atomic Energy Agency (JAEA), Japan, another physical mutagen (ion beam) has been used to create higher mutation effects especially on useful characters such as novel flower colour and pattern, pest and disease tolerance, and long flower shelf-life to meet the continuous demand of commercial growers and consumers for value-added varieties. In contrast with other physical mutagens, ion beam irradiation has been efficiently used to change target phenotypes without affecting other useful agronomic traits in the irradiated plants (Okamura *et al*., 2003; Shikazono *et al*., 2005). Among the ornamental mutants that were successfully developed through ion beam irradiations include chrysanthemum (Nagatomi *et al*., 2003), rose (Yamaguchi *et al.,* 2003), as well as petunia and torenia (Miyazaki *et al*., 2006).

Tissue culture samples from two most important cut-flower plants in Malaysia (orchid and chrysanthemum) were sent to JAEA for ion beam irradiation. Apart from the new colours of flower, other target traits

TABLE 1
Ornamental mutant varieties officially released / developed by Malaysian Nuclear Agency using acute gamma irradiation

Species/ variety	Explants	Mutagenesis type	Released mutants	New characters
Hibiscus rosa-sinensis	Cuttings	*In vivo*	Siti Hasmah PinkBeauty Siti Hasmah RedShine Nori	Pink flower colour, profuse flowering Dark red flower colour, profuse flowering Red, multiple-layer petal
Chrysanthemum morifolium	Petal cultures	*In vitro*	Nazarea Gracewhite Nazarea Softpink	White flower colour and multiple petal layer Light pink flower colour and multiple petal layer
Cordyline terminalis	Cuttings	*In vivo*	Teguh Jaguh Mantap	Dark green leaves, red stripes around the edges Deep red leaves, white stripes around the edges Light green leaves with narrow cream and red stripes
Cordylines fruticosa	Cuttings	*In vivo*	Shuhaii	Broad green leaves, dark red stripes in young leaves
Duranta repens	Cuttings	*In vivo*	Marginata Variegata	Narrow, yellow leaves with dark green lining around the edge Large, yellow leaves with various shades of green patches
Dendrobium Sonia	Protocorm-like bodies	*In vitro*	KeenaRadiant KeenaOval KeenaAhmadSobri KeenaHiengDing	Narrow and elongated petals, pale purple flower Oval shape, purple-pink petals Diamond shape petal, narrow and long lip, uniform purple colour Broad and pointed petal, pigmented veins and smudge of purple on sepals
Tradescantia spathacea	Young shoots	*In vivo*	Sobrii	Green and cream variegated upper leaf surface, reddish-purple lower surface
Petunia hybrida	Leaf discs	*In vitro*	NK Tropicana	Small, red-pink flower
Hippeastrum puniceum	Bulb scales	*In vitro*	Orange BioGamma	Bright orange flower, long leaves

were found to have extended its shelf life and insect resistance. Several potential mutants for both orchids and chrysanthemum, irradiated with this mutagen have been developed and are now being propagated to verify the stability of the new traits (Affrida *et al*., 2008; Zaiton *et al*., 2009).

Recently Nuclear Malaysia completed the development of chronic irradiation facility called Gamma Green House, and has started using this facility in the present mutation breeding programme. Chronic irradiation is an exposure to ionizing radiation over a long period (weeks or months), depending on the nature and sensitivity of the irradiated plants (Azhar *et al*., 2009). Previous studies on chronic gamma irradiation have found that chronic irradiation is very useful in minimizing radiation damage, and can induce a few improved characters on irradiated plants at the same time (Okamura, 2008). To date, several potential mutant lines of *Hibiscus rosa-sinensis* with different flower colours have been identified and they will be propagated further up to the fourth generation (M1V4) to confirm the stability of the traits.

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Seasonal Abundance of *Thrips hawaiiensis* (Morgan) and *Scirtothrips dorsalis* (Hood) (Thysanoptera: Thripidae) in Mango Orchards in Malaysia

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ABSTRACT

Investigation on seasonal abundance of mango flower thrips was carried out during a flowering season of December 2008 - March 2009 in a commercially managed mango orchard and a control orchard, where no pesticide was applied to control mango pests. *Thrips hawaiiensis* (Morgan) and *Scirtothrips dorsalis* (Hood) were the most prevalent species in the commercial and the control orchards, respectively. The highest number of adults was significantly found in flowers on the upper canopy, while more immatures were collected from the lower canopy in both orchards. Three major population peaks were discernible for the two species of thrips in this season. The population of *T. hawaiiensis* first peaked two weeks after the onset of flowering in both orchards. Meanwhile, the population of *S. dorsalis* peaked one week earlier in the commercial orchard, but the growth was slower in the control orchard, with the first peak occurring three weeks after the start of the flowering season. Abiotic factors, such as temperature and relative humidity, were found to have significantly influenced the abundance of thrips in this season. The effect of pesticides on the thrips population was also noticeable, with lower abundance recorded in the commercial orchard compared to the control orchard. The findings of this particular research can contribute in improving the management strategies of thrips in mango orchards.

Keywords: Thrips hawaiiensis, *Scirtothrips dorsalis*, seasonal abundance, mangoes

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INTRODUCTION

Mango, *Mangifera indica* Linnaeus (Anacardiaceae), known as the king of fruit (Hussain *et al.*, 2002), is one of the most consumed fruits that occupies about 4565 hectares of agricultural land areas in Malaysia (Kwee & Chong, 1994). Mango

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suffers from several pest infestations during its growth from seedling until fruit maturity. Nearly 6000 thrips species are currently recognized worldwide (Mound, 2009). Mango flower thrips infest flower panicles, which consequently reduce fruit production and fruit quality (Higgins, 1992; Pena *et al*., 2002). Among the thrips species, *Scirtothrips dorsalis* and *Thrips hawaiiensis* were recorded as severe pests of various vegetables, fruits and ornamental crops in eastern Asia (Seal *et al.*, 2006; Reynaud *et al*., 2008).

As mango is gaining popularity among growers, information on thrips population dynamics is extremely important to better manage its population. This study focused on seasonal abundance and within-plant distribution of thrips, which are crucial for the timing of insecticides application to control this pest and efficient coverage of the chemicals on the panicles. The influence of temperature and relative humidity on population density of thrips will anticipate the severity of its infestation at various ranges of these environmental parameters.

MATERIALS AND METHODS

Study Areas

The populations of *T. hawaiiensis* and *S. dorsalis* were monitored at two mango orchards in Balik Pulau, Penang; a routinely sprayed orchard with pesticides (approximately 2 ha) located at Kampung Perlis and an unsprayed orchard (approximately 0.3 ha) located at Kampung Sungai Burung. Pesticides, such as imidacloprid, cypermethrin, malathion, abamectin, chlorpyrifos and mancozeb, were applied throughout the year in the commercial orchard, but imidacloprid and cypermethrin were only applied during the flowering season.

One- to four-year predominantly MA224 (Chok Anan) mango trees were cultivated in both orchards. Mango trees were planted 4 m apart within rows, with 5.5 m between the rows in the commercial orchard and the distance between rows was 4 m, with 5 m within the rows in the control orchard. Trees were pruned regularly to 150-180 cm in height after fruit harvest to maintain their sizes mainly for ease of the next harvest. The trees were irrigated as necessary through drip tubes in the orchards.

Thrips Sampling using CO_2 Technique

The sampling of the two thrips species, *T. hawaiiensis* and *S. dorsalis* inhabiting within mango panicles, was conducted at weekly intervals during one flowering season. All the samples were collected between 1000 to 1300 hrs, according to the study conducted by Tappan (1986). The panicles from 35 trees of approximately similar size (about 170 cm high) were randomly selected. The canopy of each tree was stratified into two sections, namely, upper (>100 cm from the ground) and lower (50-100 cm from the ground) halves. One panicle was arbitrarily selected from each section. Each panicle was gently covered with a plastic bag, while the thrips within the panicles were immobilized with CO_2 (supplied by Malaysian Oxygen Berhad Sdn. Bhd. in a 25×55 cm cylinder) that was

released into the bag through a hose for 30s, and at a flow rate of 3.45 kPa (50 psi).

The time of exposure was determined based on a preliminary trial and after 30 s, most of the thrips in the panicle became inactive and fell to the bottom of the bag. The sample of each stratum was placed in a separate plastic bag, marked with the date and tree stratum. The samples were taken to the laboratory for further analysis. Temperature was recorded using a hand-held thermometer and relative humidity was estimated by a hygrometer within the orchards. In the laboratory, the plastic bags were washed thoroughly with 70% ethanol. The density and species of the thrips per panicle were also recorded.

Microscope slides were prepared based on the methodology described by Mound (2007). Specimens were identified to the species level using the key provided by Moritz *et al.* (2004). Their identification was verified by Dr. Surakrai Permkam, at the Department of Pest Management, Faculty of Natural Resources, Prince Songkla University, Hat Yai, Thailand. A series of voucher specimens were deposited at the Insect Collection Unit, the Laboratory of Entomology, Universiti Sains Malaysia, Penang, Malaysia.

Data Analysis

The mean number of thrips species collected by the CO_2 method from the two orchards was analyzed using student's t-test. The paired t-test was used to compare the densities of thrips species between the upper and lower canopy levels. The association between the thrips population and abiotic factors, such as temperature and relative humidity, was analyzed using linear regression (SPSS, 2004).

RESULTS AND DISCUSSION

Seasonal Abundance of T. hawaiiensis *and* S. dorsalis

Very high abundances of *T. hawaiiensis* (with the total of 24289 and 26490) and *S. dorsalis* (with the total of 14339 and 30721) were collected from the commercial and the control orchards, respectively. *T. hawaiiensis* was dominant in the commercial orchard during the flowering season of Dec 2008 - Mar 2009 (47.47% of the total number of the thrips collected), while *S. dorsalis* (28.02%) was the second most common species. The population of *T. hawaiiensis* grew from a density of 17.72±0.440 per panicle in late December 2008, with the first peak occurring on 8th January 2009 (35.97±1.109 per panicle), which then declined by the third week of January 2009. Its density increased from the last week of January, reaching the second peak on 29th January 2009 (51.29±1.711 per panicle), while the third peak appeared on 19th February (46.60±1.398 per panicle) (Fig.1A).

The number of *S. dorsalis* per panicle fluctuated from 11.84±0.427 to as high as 25.57±1.325 during the flowering season (Fig.1B). The population of *S. dorsalis* peaked one week after the onset of flowering on 1st January 2009. The populations of *T. hawaiiensis* and *S. dorsalis* had three peaks at both the orchards (Fig.1). The period

between the first and the second peaks in density was approximately three weeks for both species, reflecting the duration spent to complete one generation. Therefore, both the species completed two population generations during the mango flowering season of Dec 2008 - Mar 2009. Pickett *et al.* (1988) reported that generation peak is an important factor that causes fluctuations in thrips densities.

The most common species in the control orchard was *S. dorsalis,* comprising 24.82% of the total thrips collected, followed by *T. hawaiiensis* (21.40%). The first peak of *T. hawaiiensis* and *S. dorsalis* occurred on 8th January 2009 (53.63±2.496) and 15th

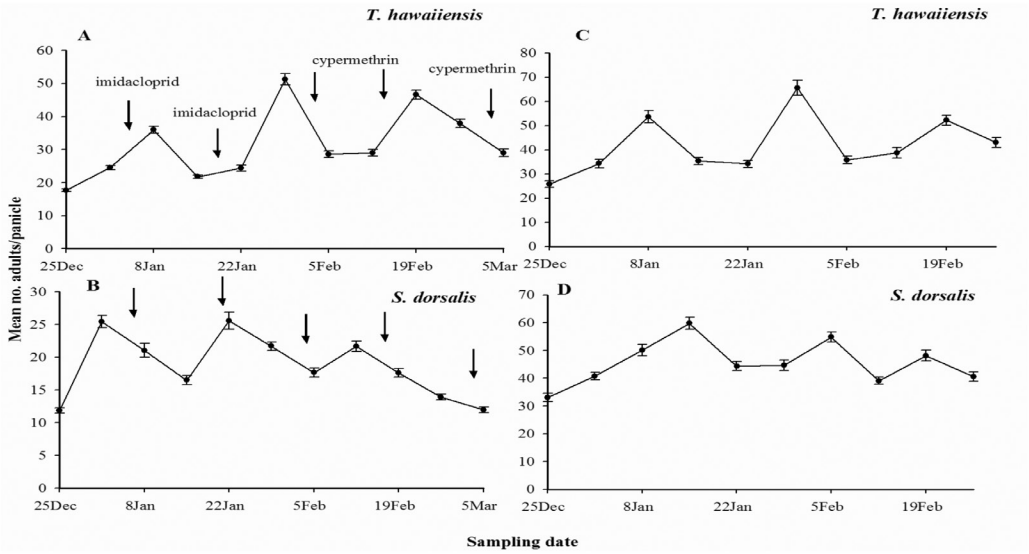

Fig.1: Seasonal abundance of adult thrips species per panicle collected from the commercial orchard (A, B) and those from the control orchard (C, D) during the flowering season (Dec 2008 - Mar 2009)

TABLE 1
The mean (±SEM) number *of the two thrips species collected from the upper and lower canopies of mango trees during the flowering season (Dec 2008 - Mar 2009) in the commercial and the control orchards

Species	Stage	Commercial orchard		Control orchard	
		Upper	Lower	Upper	Lower
T. hawaiiensis	Adult	36.45±3.684	26.63±2.690	52.66±5.798	31.11±2.090
	Instar I	6.50±0.714	8.06±1.001	12.53±1.108	16.20±1.406
	Instar II	8.68±1.169	12.22±1.391	16.47±1.019	19.71±1.100
S. dorsalis	Adult	21.03±1.841	16.21±1.269	52.53±3.407	38.55±2.046
	Instar I	4.11±0.655	5.43±0.727	13.90±1.281	17.12±1.167
	Instar II	4.88±0.597	7.46±0.910	15.98±1.125	18.22±1.138

*Values were mean per sample.

January 2009 (59.80±2.153), respectively. Both the species displayed three peaks of abundance during the flowering season, with the peaks of immatures occurring one week before those in adult abundance.

The seasonal prevalence of thrips for the first and second instar larvae in the mango orchards showed a similar distribution pattern (Fig.2 and Fig.3). Several peaks of occurrence were indicated during the flowering season. The density of larvae within the mango panicles was low in comparison with that of the adults. The declining proportion of larvae in the late flowering season indicates a declining rate of reproduction among females. Fecundity

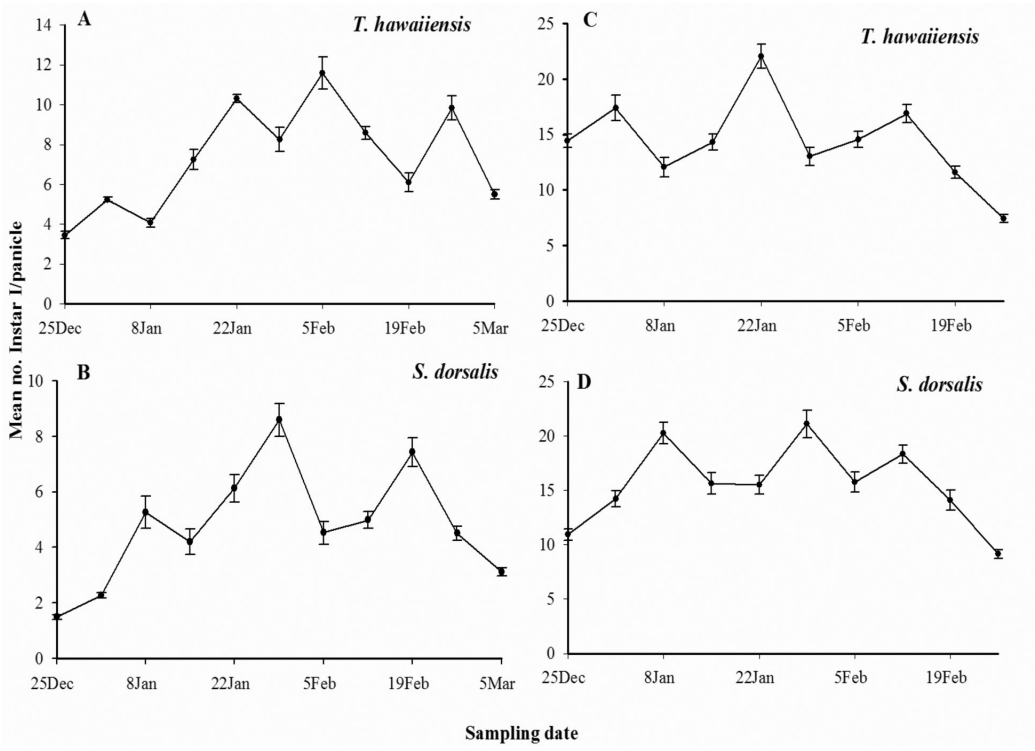

Fig.2: Seasonal prevalence of the first instar larvae per panicle in the commercial mango orchard (A, B) and those in the control orchard (C, D) during the flowering season (Dec 2008 - Mar 2009)

TABLE 2
The mean percentage* of the adult thrips in the upper and lower canopies of mango trees during the flowering season (Dec 2008-Mar 2009) in the commercial and the control orchards

Species	Commercial orchard		Control orchard	
	Upper	Lower	Upper	Lower
T. hawaiiensis	57.784	44.216	62.864	37.136
S. dorsalis	56.466	43.534	57.678	42.322

*Values were mean per sample.

depended on the consumption of pollen grains from flowers, which decreased in numbers in the late season (Pickett *et al*., 1988). Findings of the current study showed the proportion of larva to adult was higher in the control orchard than the commercial orchard. This could be attributed to a higher susceptibility of the larvae to insecticides application compared to the adults.

Meanwhile, the mean number of *T. hawaiiensis* (adults: F=0.16, df=19, P<0.001 and larvae: F=4.02, df=19, P<0.001) and *S. dorsalis* (adults: F=1.80, df=19, P<0.001 and larvae: F= 1.43, df=19, P<0.001) differed significantly between the commercial and the control orchards, implicating that chemical sprays did affect the populations of thrips. The results also indicated that *S. dorsalis* was more susceptible to insecticides pressures as its population was approximately 2.5 folds higher in the control orchard (Table 1).

Within Plant Distributions of T. hawaiiensis and S. dorsalis

Adults of both species were significantly more prevalent in the upper canopy of mango trees than in the lower canopy, while more larval thrips were found in the lower part of the canopy in both orchards (Paired

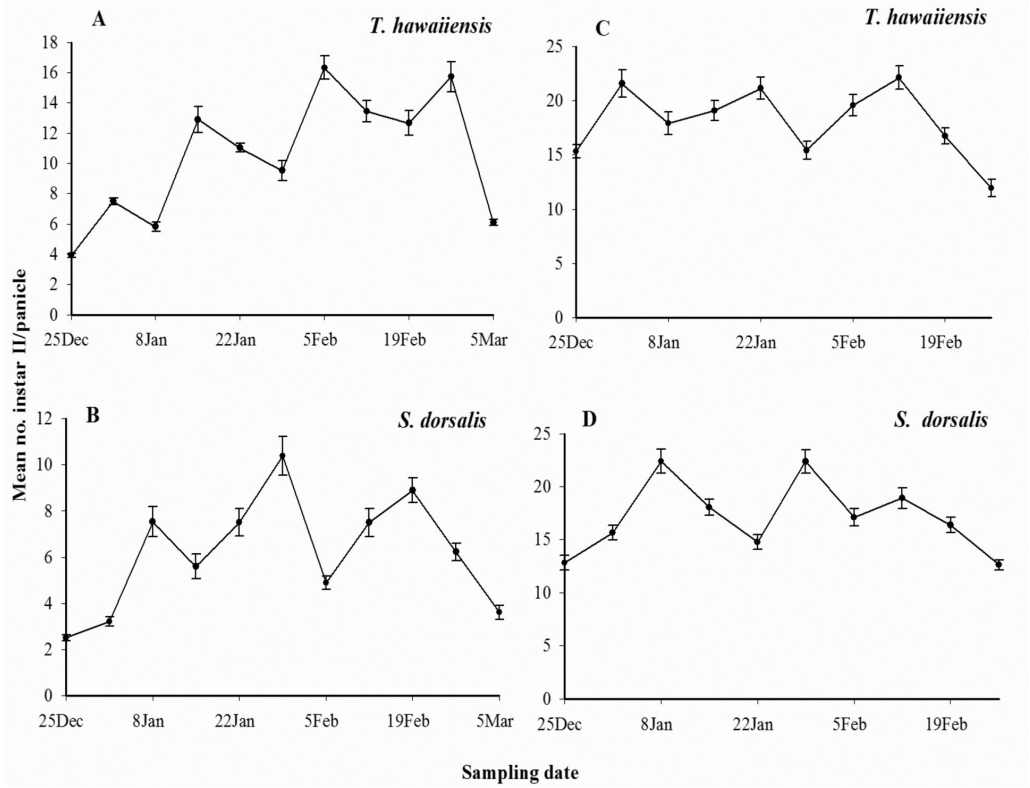

Fig.3: Seasonal prevalence of the second instar larvae per panicle in the commercial mango orchard (A, B) and those in the control orchard (C, D) during the flowering season (Dec 2008 - Mar 2009)

t-test, all $P<0.05$, respectively). However, the patterns of distribution were very similar in the two canopy levels (Table 1).

Salguero-Navas *et al*. (1991) reported higher numbers of *Frankliniella occidentalis* (Pergande) and *F. tritici* (Fitch) in the upper canopy of tomatoes than in the lower flowers at the commercial tomato fields. The opposite was true for immatures, with more being collected from the lower flowers. Reitz (2002) reported similar results for *F. bispinosa* (Morgan). Increased activity of thrips within the higher canopy of plants was also reported by Broadsgaard (1989) and Gillespie and Vernon (1990). The results of the present study indicated that the higher percentage of adult thrips was found in the upper canopy in the control orchard compared to the commercial orchard (Table 2). This result could be explained as adults near the top were more susceptible to frequent insecticides application than those located in the lower canopy.

Effects of Temperature and Relative Humidity on the Population of Thrips

A linear regression analysis resulted in a positive slope showing a positive relationship between the mean number of both thrips species per panicle and temperature in both orchards (*T. hawaiiensis*; commercial: $F=11.13$, $df=1,9$; $P=0.009$, y=4.74x-114.72 and control: $F=7.09$, $df=1,8$, $P=0.02$, y=5.74x-141.38 and *S. dorsalis*; commercial: $F=6.50$, $df=1, 9$, $P=0.03$, y=1.93x-40.99 and control: $F=3.92$, $df=1,8$, $P=0.03$, y=3.20x-56.74). The density of thrips appeared to increase within the mango panicles with increased temperature, but the population densities of *Thrips hawaiiensis* (commercial: $F=6.24$, $df=1,9$, $P=0.03$, y=-0.75x+92.38 and control: $F=2.68$, $df=1,8$, $P=0.03$, y=-0.76x+102.39) and *S. dorsalis* (commercial: $F=1.04$, $df=1,9$, $P=0.04$, y=-0.18x+32.93 and control: $F= 1.03$, $df=1,8$, $P=0.04$, y=-0.07x+40.22) were negatively associated with relative humidity, which is in agreement to Bagle (1993) and Wahla *et al*. (1996) who reported that relative humidity had a negative relationship with the thrips population.

CONCLUSIONS

Identifying the species composition of thrips inhabiting within mango panicles and determining the temporal pattern of thrips population during flower development will lead to improve sampling protocols and management plans for flower thrips. Large populations of *T. hawaiiensis* and *S. dorsalis* within mango panicles in both orchards have provided evidence that these two thrips species were responsible for cosmetic injuries observed on the mango fruits at these orchards.

In particular, more adults (of both species) were found in flowers on the upper canopy, while more immatures were collected from the lower canopy. This information is important for selecting the most appropriate sampling unit to estimate the densities of the adult and immature and for effective insecticides application. Three major population peaks were observed for the two thrips species in one season at both the orchards. Determining the peak of

thrips abundance within the mango panicles ensures effective timing for sampling and controlling this particular pest at mango orchards.

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Isolation of Metal Tolerant Bacteria from Polluted Wastewater

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ABSTRACT

Screening of standing and flowing water sample from Malaysian gold mine environment yielded 24 single colonies and all isolates were assessed for their metal tolerance capability. A preliminary screening on Chloride Free Medium (CFM) agar plate supplemented with 5mM of Cu^{2+}, Ag^{+} and Zn^{2+} showed that two isolates were tolerant towards Cu^{2+} ion, while two other isolates were tolerant towards Zn^{2+} ion and one single isolate was tolerant towards Ag^{+} ion. Partial identification by 16S rRNA determined that they are only two distinct species of bacteria, namely, *Bacillus* sp. and *Achromobacter* sp. The identification was supported by physical and biochemical characterizations which showed that *Bacillus* sp. was a positive rod while *Achromobacter* sp. was a gram negative coccus. Maximum tolerance concentrations (MTC) of *Bacillus* sp. and *Achromobacter* sp. were determined in liquid CFM medium and the results showed that *Bacillus* sp. could tolerate up to 20 µM Cu^{2+} ion and 2.5 mM Zn^{2+} ion, while *Achromobacter* sp. could tolerate up to 5 µM Ag^{+} and 20µM Cu^{2+} ion.

Keywords: Bacteria, Metal ion, Metal tolerant bacteria, Mining environment

INTRODUCTION

With the advancement of industrial development, environmental pollution caused by toxic heavy metals is increasingly becoming an ecological risk. Heavy metal pollution in the environment can occur naturally and it is caused by leaching of metals from soils. Effluent discharge from mining activity is another reason of accumulation of metals in water sources in Malaysia (DOE, 1997). Acid mine drainage (AMD) produced during mining activity could leach out heavy metals such as mercury, lead and arsenic from the waste ore and carried downstream as water washes over the rock surface (Corpwatch, 2007).

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This situation can cause concentration of heavy metals in mine areas to escalate up to 50 g/kg, depending on the type of metals and area of contamination (Monica, 2008).

In this work, Cu^{2+}, Zn^{2+} and Ag^{+} ions were chosen for the metal tolerance study on isolated bacteria. These three metal ions were chosen because of their differences in the toxicity level to microbial cells. Zn^{2+} ion is an essential metal ion as it serves as a micronutrient as well as a component on zinc-finger protein inside the bacteria cell (Tan, 2007; Abskharon *et al.*, 2008). On the other hand, Cu^{2+} is a non-essential metal ion where only low concentration of Cu^{2+} is needed in the cell for the activity of the enzymes to occur (Ryu *et al.*, 2003; Yu *et al.*, 2009). Both Zn^{2+} and Cu^{2+} can enhance microbial growth at low concentrations but suppress growth at high concentrations. In contrast, Ag^{+} is a toxic metal ion which can cause changes in the physiology and biochemistry of the cell at a concentration as low as 20 µM (Ratte, 1999; Slawson *et al.*, 1990).

Microorganisms are always the first biota to be contacted with metal pollution. The interaction between the microorganisms with metals has been well documented (Hughes & Poole, 1989; Slawson *et al.*, 1990). Biological organisms are easily affected directly or indirectly by heavy metal pollution. However, there are reports on the adaptation of microorganisms towards heavy metals that make them innocuous (Ibrahim, 1993; Yu *et al.*, 2009). Metal ions affect microorganism by reducing their growth and activity which can be reflected by a reduction of the growth rate and an increase in lag time (Gikas *et al.*, 2009). To survive under metal ions stress conditions, microorganisms have evolved several defence mechanisms either by quick and unspecific or slow and substrate specific (Spain & Alm, 2003). They respond to heavy metal stress using different defence systems, such as excluding metal ions from the cell, reducing to a less complex compounds, forming a complex by thiol-containing molecules and synthesizing metal binding proteins (Slawson *et al.*, 1990; Neis, 1999; Malin & Leif, 2001; Hussein *et al.*, 2004).

It is important to note that a sound knowledge of the interactions between microorganisms and metal species is fundamental to understanding the behaviour and fate of trace metals in the environment. Thus, the aims of this study were to isolate and identify indigenous bacteria from contaminated mining area and to assess their tolerance towards the toxic levels of Ag^{+}, Zn^{2+} and Cu^{2+} ions. For this purpose, bacteria were isolated from contaminated water points in the mining area. The isolates were then screened for their ability to grow on minimal media supplemented with 5mM of metal ions under investigation. The selected isolates were then characterized and identified using the biochemical and molecular biology technique. The metal tolerance capabilities of the bacteria isolates were further investigated through a maximum tolerance concentration (MTC) study. It is hoped that the results from this study can be a precursor to understanding

the interactions of microorganisms with heavy metals and will eventually lead to the development of tools for the detection of the level of metals in the environment, as well as facilitate in the *in situ* decontamination of metal-polluted waste sites.

In this study, the wastewater sample was collected from a Malaysian gold mine environment which was contaminated with approximately 5mg/l of copper and less than 1mg/l of zinc in the drainage (Natural Environment Research Council, 1995). The aim of this study was to isolate indigenous bacteria that are tolerant towards silver (Ag^{+}), zinc (Zn^{2+}) and copper (Cu^{2+}) ions that exist in the gold mine. The isolated bacteria were characterized physiologically and biochemically, as well as were tested for their degree of metal tolerance.

MATERIALS AND METHODS

Description of the Site

Penjom gold mine (4° 8' 25'' N, 101° 59' 6'' E), which is located in Peninsular Malaysia covers an area of 8.199 km^2 (2,026 acres), and was selected as a sampling location for this research. The samples were collected from different points surrounding the gold mining area. The area has a tropical climate with an annual temperature of about 20.1 to 31.8°C. Fig.1 shows the main location of the sampling site.

Fig.1: Location of the sampling site

Sample Collection

The water samples from the Malaysian gold mine environment were collected in screw-capped bottles. *In-situ* water characterizations for the pH, temperature and dissolved oxygen percentage were recorded during the wastewater collection.

Preparation of the Solutions and Media

All glassware was autoclaved prior to use. All the chemicals used were of Analar grade or equivalent and dissolved in distilled water. 100 mM of heavy metal stock solution of Zn^{2+}, Cu^{2+} and Ag^{+} were prepared by dissolving $ZnSO_4.7H_2O$, $CuSO_4.5H_20$ and $AgNO_3$ respectively in distilled water and sterilized by filtration using 0.20µm pore size (Whatman) (Sabry *et al.*, 1997; Van Nostrand *et al.*, 2007).

Bacteria Isolation and Culture Conditions

Nutrient broth (NB) was inoculated with 10% v/v water sample from the Malaysian gold mine environment and incubated at 30°C, 200 rpm. After overnight incubation, these bacterial cultures were serially diluted in distilled water (10^{-3} to 10^{-7}) before they were spread on nutrient agar (NA). The plates were incubated at 30°C for 1 day. The colonies with different morphological appearances were selected and further subcultured on the same media. All the growing bacteria cultures were stored at -80°C in 20% glycerol.

Preliminary Screening for Metal Tolerance

Each isolated culture was tested for metal tolerance by growing it on slightly modified Chloride Free Medium (CFM) agar plate (Ahmad, 1998; Ibrahim, 2003). This medium contained the following chemical reagents: Tris (4mM), $K_2HPO_4.3H_20$ (2.8mM), $KH_2PO_4.3H_20$ (2.2mM), NH_4NO_3 (18.7Mm), $CaSO_4$ (0.001Mm), K_2SO_4 (2.0mM), $MgSO_4.7H_2O$ (1.0mM) and glycerol (5g per litre). The final pH was adjusted to 7.0 to 7.4. Then, 10 ml of the following trace element solutions (pH 7-8) were added into the solution (g/l): $Na_2EDTA.2H_20$ (5.0), $Fe_2(SO_4)_3$ (0.37), $Co(NO_3)_2.6H_2O$ (0.01), ZnO (0.05), $CuSO_4.5H_20$ (0.015), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.01) and H_3BO_3 (0.01g). Meanwhile, 10% of the agar powder (Analar grade or equivalent) was added into the agar plates. This is followed by supplementing 5mM of the investigated metal ions into the medium. Ag^{+} ion was added as $AgNO_3$, while Cu^{2+} ion was added as $CuSO_4.5H_20$ and Zn^{2+} ion was added as $ZnSO_4.7H_2O$.

Maximum Tolerance Concentration (MTC) Study

Determination of maximum tolerance towards metal ions of bacterial isolates was registered on CFM agar plates, followed by agar dilution method described by Hassan *et al*. (2008). Each agar plate was supplemented with 1-14mM Cu^{2+}, Zn^{2+} and Ag^{+} ion. The plates were inoculated with the grown isolates and incubated at 30°C for 7 days (Ibrahim, 1993).

Metal Tolerance Experiment

The improvement of the tolerance level of isolates on the increasing concentrations

of Cu^{2+}, Zn^{2+} and Ag^{+} was carried out in CFM liquid medium, followed by the serial transfer method proposed by Ibrahim (1993). The bacterial isolates that grew in the CFM liquid medium in the absence of metal ions were used as the starter cultures. This was followed by subculture into fresh CFM medium that was supplemented with 1µM of Cu^{2+}, Zn^{2+} or Ag^{+}. The cultures were incubated at 30°C, shaken at 200 rpm and the growth of the bacteria was monitored based on turbidity using Jenway spectrophotometer (wavelength: 600nm). After the bacterial culture had reached its exponential phase, it was further subcultured into the CFM liquid medium supplemented with higher concentrations of Cu^{2+}, Zn^{2+} or Ag^{+}. This procedure was repeated with increasing concentrations of metal ions until the MTC of the metal ions was reached. The bacterial cultures inoculated into CFM broth without addition of metal ions acted as a control.

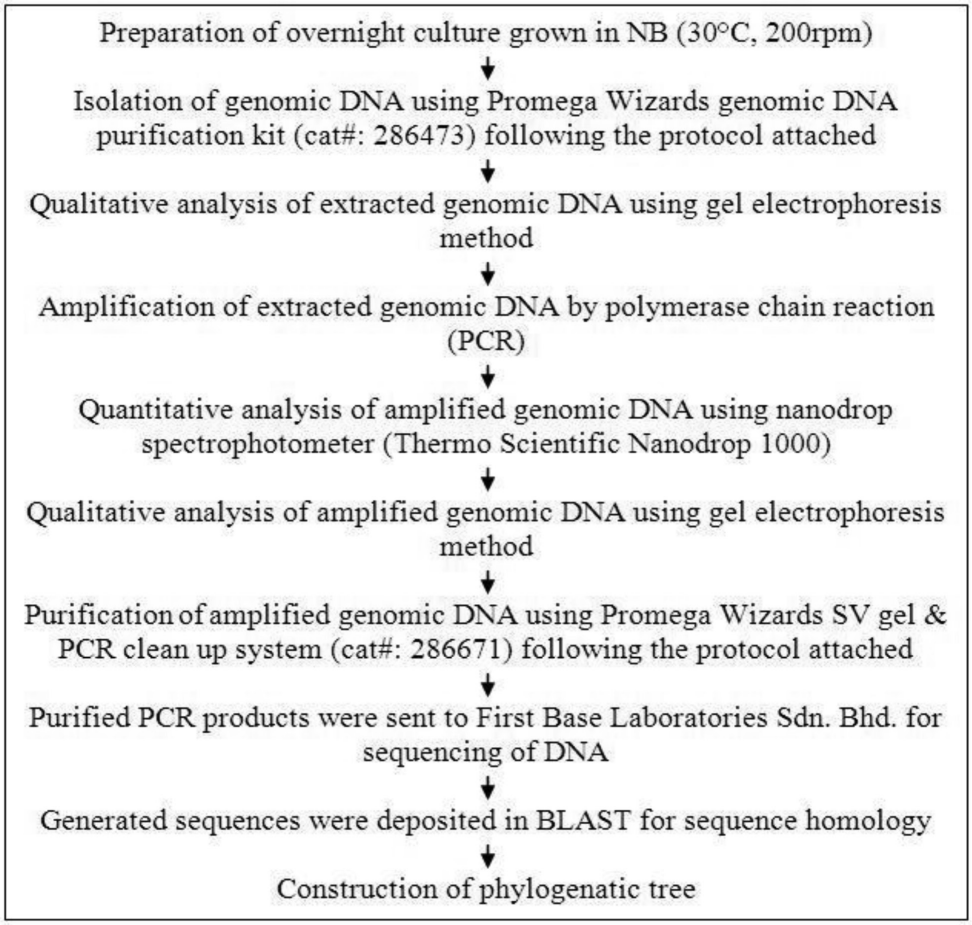

Fig.2: Partial identification using 16S rRNA method of metal tolerant bacteria

Physical Characterization of the Metal Tolerant Bacteria

The isolated bacteria were physically characterized by using a standard gram staining method to identify gram positive and gram negative bacteria (Libman *et al.*, 2006; Brown, 2007; Wan Mohd Azemin, 2010).

Biochemical Characterization of Metal Tolerant Bacteria

Further characterization of the bacterial isolates was carried out via biochemical test method, as described in the book entitled, *"Biochemical Test of Medical Bacteria"* by MacFaddin (1980).

Phylogenatic Study of Metal Tolerant Bacteria

Bacteria that showed some tolerance towards Cu^{2+}, Zn^{2+} and Ag^{+} metal ions were partially characterized using 16S rRNA. The use of 16S rRNA gene sequences is very important as a common housekeeping genetic marker because of its ability to provide bacterial identification up to species level. In this study, 16S rRNA steps were carried out following the method by Yan (2008) with slight modifications. The overall step for the 16S rRNA technique is shown in Fig.2.

An overnight culture of Cu^{2+}, Zn^{2+} and Ag^{+} tolerant bacteria in NB was prepared for the extraction of genomic DNA. The extraction of the genomic DNA was carried out using Promega Wizard DNA extraction kit following the instructions recommended by the supplier prior checked of extracted DNA on agarose gel (1% of agarose in TAE buffer) for one hour at 80mV (Ziegler *et al.*, 2007). In order to study the evolution of Cu^{2+}, Zn^{2+} and Ag^{+} tolerant bacteria, the extracted DNA gene was amplified using two sets of oligonucleotide primer. The first set was forward primers 5'-AGAGTTTGATCCTGGCTCASG-3' and reverse primer 5'-AAGGAGGTGATGCAGCC-3', while the second set was forward primer 5'- AGAGTTTGA CCTGGCTCAG-3' and reverse primer 5'-AAGGAGGTGAATCCAGC-3' using Biorad MJ mini thermocycler. The PCR reaction mixture and its condition are presented in detail in Tables 1 and 2.

TABLE 1
PCR reaction mixture

Reagents	Volume (µl)
Extracted DNA	5
Forward primer	1
Reverse primer (pH)	1
PCR master mix	25
Nucleas free water	18
Total	50

TABLE 2
PCR cycling profile

PCR steps	Temperature (°C)	Duration (min)
Initial denaturation	94	4
Denaturation (30 cycles)	94	1
Annealing (30 cycles)	50	1
Extension (30 cycles)	72	4
Final extention	72	10

The PCR products were subsequently cleaned with Promega Wizards SV gel and PCR clean up system prior to qualification

on agarose gel electrophoresis in the same conditions. 50µl of the amplified DNA, with an approximate concentration of 4ng/µl, was sent for DNA sequencing at First Base Laboratories Sdn. Bhd. All the generated sequences were deposited in the GeneBank database through BLAST (Basic Local Alignment Search Tool) which could be accessed at www.ncbi.nlm.nih.gov/BLAST for sequence homology before phylogenetic tree construction. Multiple sequence alignment was carried out using Sequence Scanner v1.0, while the construction of phylogenetic tree was performed using CLC Sequence Viewer 5.1.2 by making use of the generated sequence using bootstrapping and neighbour-joining methods.

RESULTS AND DISCUSSION

Physico-chemical Properties of the Sampling Sites

Mining-based environment is one of the sources of metal pollution into the environment. The temperature of the Malaysian gold mine environment was measured to be approximately 30.37°C, while the pH was around 7.76 with a dissolved oxygen percentage value of 5.5%. This information is important as it gives data on the most suitable parameters, primarily the temperature and pH to be used for the growth of bacterial cultures in a laboratory setting.

Isolation of the Indigenous Bacteria from the Malaysian Gold Mine Environment

The estimation of the total bacterial population present in the water sample was found to range from 200- 400 colonies/100 ml at different sampling points. A total of 24 single colonies were isolated and preserved in 20% glycerol and stored at -80°C for further studies. Table 3 lists out the number of the isolated bacteria from the standing and flowing water samples of the Malaysian gold mine environment.

TABLE 3
The number of isolated bacteria from the standing and flowing water samples of the Malaysian gold mine environment

Water samples	Number of bacteria
Standing water samples	1
Flowing water samples	23
Total isolated bacteria	24

Preliminary Screening of Metal Tolerant Bacteria

All the isolates were tested for heavy metal ion tolerance on 15 ml of CFM agar plate supplemented with 5mM of Cu^{2+}, Zn^{2+} and Ag^{+} metal ions. Ag^{+} ion was added as $AgNO_3$, while Cu^{2+} ion was added as $CuSO_4.5H_20$, and Zn^{2+} ion was added as $ZnSO_4.7H_2O$. The number of the grown bacteria and the percentage of the tolerated bacteria to Cu^{2+}, Zn^{2+} and Ag^{+} metal ions are shown in Table 4. The results showed that two isolates were tolerant towards Cu^{2+} ion, while two other isolates were shown to be tolerant towards Zn^{2+} ion, and one isolate was tolerant towards Ag^{+} ion. The concentration of 5mM metal ions is considered high for screening purpose so as to identify the bacteria that can tolerate metal ions. In her research, Ibrahim (1993) supplemented only 5- 200µM of Ag^{+} ion

to screen *Pseudomonas diminuta* and *Aeromonas hydropresearcherhila*. In another study, Piotrowska-Seget *et al.* (2005) used up to 5mM Cu^{2+} and Zn^{2+} ion as well as 0.5mM Ag^{+} ion amended in nutrient agar to count metal tolerant population whereas the used of rich medium might contribute to the growth of bacteria. In this study, the used of minimal medium supplemented with metal ions gave the maximum bioavailability of metal ions to the bacteria culture.

TABLE 4
The number of isolates growing on CFM agar medium supplemented with 5mM of Cu^{2+}, Zn^{2+} and Ag^{+} metal ions

Metal ion	Number and percentage of bacteria tolerant towards 5mM metal ion	Bacteria designation
Cu^{2+}	2* (8.3)	32E4, 32F5
Ag^{+}	2* (8.3)	22D2, 21H1
Zn^{2+}	1* (4.17)	11F1

*Values indicated the number of tolerant isolates
Values in the parentheses indicated percentage of tolerant isolates

Determination of the Maximum Tolerance Concentration (MTC) of Metal Tolerant Bacteria

The two isolates shown to have grown on the CFM agar plates supplemented with 5 mM Cu^{2+} ion were designated as 32F5 and 32E4, while two other Zn^{2+} tolerant isolates were designated as 22D2 and 21H1 and a single isolate that grew on CFM agar plate supplemented with 5mM Ag^{+} ion was designated as 11F1 (see Table 4). These five bacteria were streaked on the CFM agar plate supplemented with 1-14 mM of respective metal ions to further investigate their degrees of tolerance towards metal ions. The growth pattern on the individual isolates towards Cu^{2+}, Zn^{2+} and Ag^{+} metal ions is shown in Table 5.

The results in Table 5 show that all the bacteria grew on the CFM agar medium in the absence of metal ions. Bacteria 21H1 was found to be able to tolerate Zn^{2+} ion up to 13mM while bacteria 22D2 tolerated up to 10mM Zn^{2+} ion concentration. Meanwhile, 21H1 was shown to grow faster than 22D2 and 21H1 grew on the CFM agar

TABLE 5
The growth pattern of Cu^{2+}, Zn^{2+} and Ag^{+} tolerant isolates

Heavy metal concentration (mM)		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Zn^{2+}	22D2	+	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{6}$	$+^{7}$	$+^{7}$	$+^{7}$				
	21H1	+	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{4}$	$+^{5}$	$+^{6}$	$+^{6}$	$+^{6}$	$+^{7}$	$+^{7}$	$+^{7}$	
Cu^{2+}	32E4	+	$+^{4}$	$+^{7}$												
	32F5	+	$+^{5}$	$+^{7}$												
Ag^{+}	11F1	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Subscript number showed the days needed for heavy metal bacteria to grow on the CFM agar plate added with heavy metal ion
*: Bacterial growth cannot be observed

plate supplemented with 7mM Zn^{2+} ions in 5 days and easily grew up to 13 mM concentration within 7 days. The growth of 22D2 was found to be much slower and it tolerated lower concentrations of Zn^{2+}. The highest Zn^{2+} ion concentration that it could be tolerated was 10mM. In contrast, the bacteria that grew on the CFM agar plate added with Cu^{2+} ion could grow in the Cu^{2+} ion concentration of up to 2mM. Both Cu^{2+} tolerant 32E4 and 32F5 grew slower on the CFM agar plate added with Cu^{2+} ion, in which 32E4 only started to grow on day 4, while 32F5 started to grow on day 5.

Physical and Biochemical Characterization of Metal Tolerant Bacteria

Cu^{2+} tolerant 32E4 and 32F5, Zn^{2+} tolerant 22D2, whereas 21H1 and Ag^{+} tolerant 11F1 were characterized based on the colony and cell morphology as well as the biochemical and gene analysis. The physical and biochemical characterization of the metal tolerant bacteria were determined according to the book by MacFaddin (1980) entitled, "Biochemical Tests for Identification of Medical Bacteria."

Physical Characterization of the Isolates

Colony morphology

Ag^{+} tolerant isolate, 11F1, was found to be creamy white in colour and round in shape, while the elevation and margin of the colony were entire and raised. Zn^{2+} tolerant bacteria 21H1 displayed a similar colony morphology with Ag^{+} tolerant bacteria, 11F1. Cu^{2+} tolerant bacteria, 32F5 and Zn^{2+} tolerant bacteria 22D2 shared the same colony morphology characteristics where colony colour, colony margin and colony elevation were creamy white, raised and undulate, respectively. They both differ in terms of the shape of the colony where 22D2 was wavy, and 32F5 was round. Meanwhile, Zn^{2+} tolerant bacteria (21F2) was easily distinguishable because of its colour, which is yellow. It was also found to be round in shape and acquire raised margin and entire colony elevation. Cu^{2+} tolerant bacteria (32E4) was creamy yellow in colour and round in shape, while the colony margin and colony elevation were raised and undulated. A detailed result for the colony morphology of each bacterial isolates is shown in Table 6.

TABLE 6
The Colony morphology of metal tolerant bacteria

Characteristics	Metal tolerant bacteria				
	11F1	22D2	21H1	32F5	32E4
Colony colour	Creamy White	Creamy White	Creamy White	Creamy White	Creamy Yellow
Colony shape	Round	Wavy	Round	Round	Round
Colony margin	Raised	Raised	Raised	Raised	Raised
Colony elevation	Entire	Undulate	Entire	Undulate	Undulate

Cellular morphology

Cu^{2+} tolerant 32E4 and 32F5 were found to be both gram positive bacteria and rod shaped. Ag^{+} tolerant bacteria, 11F1, was found to be a gram negative cocci, while 22D2 which was a Zn^{2+} tolerant bacteria was found to be a gram positive rod. Zn^{2+} tolerant bacteria 21H1 is a gram negative cocci. Details of the cellular morphology and Gram reaction are given in Table 7.

Biochemical Characterization of the Isolates

The biochemical characterization of metal tolerant bacteria was carried out in order to identify the bacterial isolates. In this work, the biochemical tests were carried out according to the methods by MacFaddin (1980). In this work, Mac Conkey agar was used to differentiate the gram positive and gram negative bacteria in which bile salt that contained in the agar would completely inhibit the growth of the gram positive bacteria. Zn^{2+} tolerant bacteria 22D2 and Cu^{2+} tolerant bacteria 32E4 and 32F5 were completely inhibited on the Mac Conkey agar plate. This result confirmed that 22D2, 32E4 and 32F5 were gram positive bacteria. Nevertheless, Ag^{+} tolerant bacteria and Zn^{2+} tolerant bacteria 21H1 did not grow on Mac Conkey agar plate and this indicated that they were gram negative bacteria. Lactose fermenting bacteria could also be distinguished using Mac Conkey agar plate. Both Ag^{+} tolerant bacteria 11F1 and Zn^{2+} tolerant bacteria 21H1 turned the Mac Conkey agar plate to yellow and this showed that they were non-lactose fermenting bacteria. All the metal tolerant bacteria were found to be motile where they migrated from the stab point to diffuse into the medium and caused turbidity. The Oxidation-fermentation test (OF test) was performed using commercial oxygen-fermentation medium (Difco). The Ag^{+} tolerant bacteria 11F1 and Zn^{2+} tolerant bacteria 21H1 were found to be able to metabolize a carbohydrate under aerobic condition. In contrast, Zn^{2+} tolerant bacteria 22D2 and Cu^{2+} tolerant bacteria, 32F5 and 32E4 were fermentative bacteria which could utilize carbohydrate in the absence of oxygen. The oxygen requirement test showed that all the metal tolerant bacteria were obligate aerobes which strictly need oxygen to grow. Commercial Christensen urease agar was used to identify the bacteria with the ability to split urea into ammonia. Ag^{+} tolerant bacteria 11F1 and Zn^{2+} tolerant bacteria 22D2 were found to have the ability

TABLE 7
The Cellular morphology of metal tolerant bacteria

Metal tolerant bacteria					
Characteristics					
	11F1	22D2	21H1	32F5	32E4
Gram stain test	Negative	Positive	Negative	Positive	Positive
Cell morphology	Cocci	Rod	Cocci	Rod	Rod

TABLE 8
The Biochemical characteristics of metal tolerant bacteria

Characteristics	Metal tolerant bacteria				
	11F1	22D2	21H1	32F5	32E4
Mac Conkey agar plate test	+	-	+	-	-
Lactose ferment test	-	*	-	*	*
Motility test	Motile	Motile	Motile	Motile	Motile
Oxidation- Fermentation test	O	F	O	F	F
Oxygen requirement test	Obligate aerobes	Obligate aerobes	Obligate aerobes	Obligate aerobes	Obligate aerobes
Christensen urease test	+	+	-	-	-

*No growth observed

to produce two molecules of ammonia from urea compound. Table 8 shows detailed results of the biochemical characterization of metal tolerant bacteria.

However, the results of the biochemical tests that were carried out in this work were not sufficient enough to identify the isolates accurately. Thus, 16SrRNA analysis was performed as it gives a better degree of accuracy for bacterial identification.

16S rRNA Gene Sequence Analysis

The gene sequence analysis was performed to further identify the bacteria and to support the results from the biochemical analysis. Fig.3 and Fig.4 show details of the constructed phylogenatic tree. The phylogenatic analysis of 11F1 and 21H1 showed that they are closely related (88% of bootstrap replication) to *Achromobacter piechaudii* strain Shan11. originated from *Alcaligenaceae bacterium* JS 8 (89% of bootstrap replication). On the other hand, the gene sequence analysis of Cu^{2+} tolerant bacteria 32E4 and 32F5 and Zn^{2+} tolerant bacteria 22D2 showed that they are from *Bacillus* genus and shared 100% of bootstrap replication with *Bacillus anthracis* strain V11DMK. Table 9 shows a summary of the bacterial species and metal ions that they could tolerate.

TABLE 9
The Identified species of $Cu^{2+,}$ Zn^{2+} and Ag^{+} tolerant bacteria

Metal tolerant bacteria	Metal ions	Identified species
11F1	Ag^{+}	*Achromobacter* sp.
22D2	Zn^{2+}	*Bacillus* sp.
21H1	Zn^{2+}	*Achromobacter* sp.
32F5	Cu^{2+}	*Bacillus* sp.

Metal Tolerance Experiment

Bacillus sp. and *Achromobacter* sp. were identified to be tolerant towards Cu^{2+}, Zn^{2+} and Ag^{+} ions. In this work, *Bacillus* sp. could tolerate Cu^{2+} ion and Zn^{2+} ion while *Achromobacter* sp. could tolerate Ag^{+} and Zn^{2+} ion. The results shown in Table 10

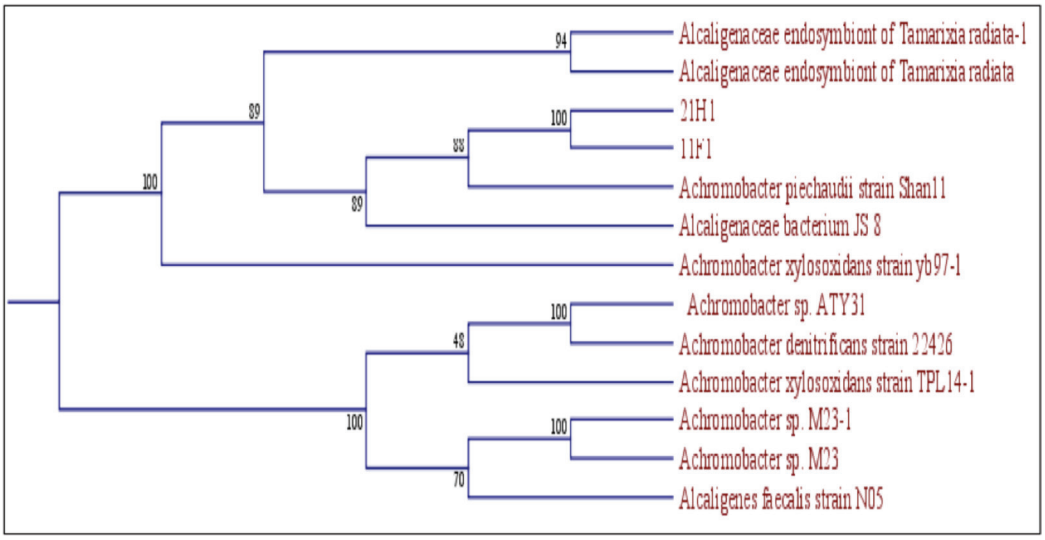

Fig.3: The Phylogenatic tree of Ag^{+} tolerant bacteria, 11F1 and Zn^{2+} tolerant bacteria 21H1

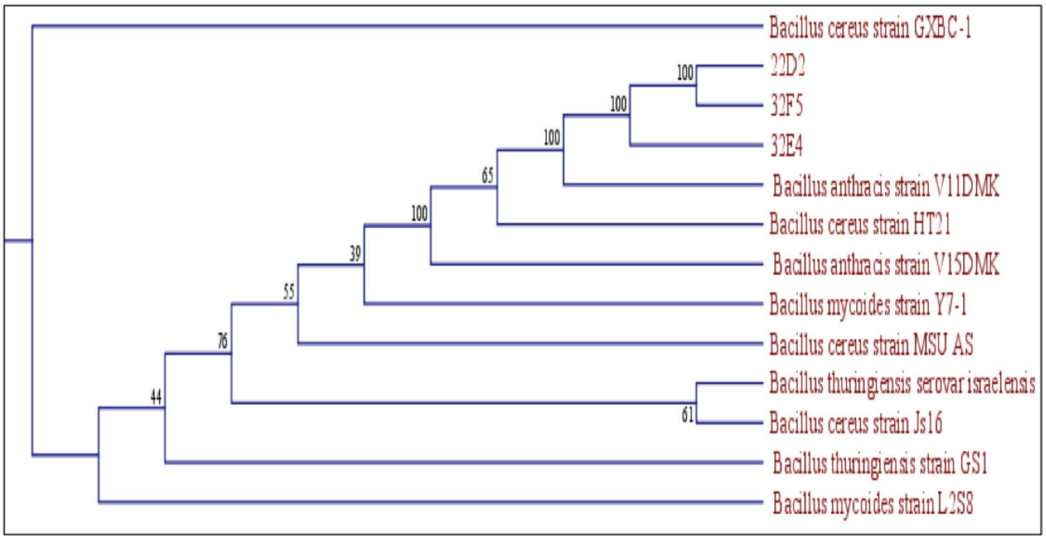

Fig.4: The phylogenatic tree of Cu^{2+} tolerant bacteria, 32E4 and 32F5 and Zn^{2+} tolerant bacteria 22D2

indicate that *Bacillus* sp. could tolerate up to 2.5mM Zn^{2+} ion and 0.02mM Cu^{2+} ion. On the other hand, *Achromobacter* sp. could tolerate 0.01mM Zn^{2+} ion and 0.005mM Ag^{+} ion. The tolerant value appeared to be lower than those of the other reports which had claimed that *Bacillus cereus* and *Bacillus thurengiensis* could tolerate 150µM and 0.2mM Cu^{2+} ion, respectively, and *Achromobacter* sp. was found to be able to tolerate Zn^{2+} ion up to 20µM (Hassen *et al.*, 1997; Raja *et al.*, 2006). However, in some previous studies, complex media was used for the metal tolerance studies; in

this study, minimal media (i.e. CFM) was used. The use of the minimal medium in this work reduced the negatively charged ion like chloride which prevents metal ion precipitation, and hence giving the maximum bioavailability of metals to the bacteria.

TABLE 10
The maximum tolerance concentrations (MTC) of *Bacillus* sp. and *Achromobacter* sp

Bacteria species	Metal ion	Maximum tolerance concentration (MTC) (mM)
Bacillus sp.	Cu^{2+}	0.02
	Zn^{2+}	2.5
Achromobacter sp.	Ag^{+}	0.005
	Zn^{2+}	0.01

Bacillus sp. and *Achromobacter* sp. grown in the CFM liquid medium in the absence of metal ions worked as a control. Fig.5 and Fig.6 show the growth curve of *Bacillus* sp. and *Achromobacter* sp. in the CFM liquid medium in the absence of metal ions. Both the bacteria showed lengthy log phase, especially *Achromobacter* sp. which took approximate 150 hours before entering lag phase whereas *Bacillus* sp. took about 30 hours. This might be due to the use of the minimal medium for growth.

CONCLUSION

A study on the isolation of bacteria from a Malaysian gold mining environment yielded 5 bacterial isolates that were later identified to be only 2 distinct species, namely, *Bacillus* sp. and *Acromobacter* sp. Zn^{2+} tolerant bacteria have the ability to tolerate up to 2.5mM Cu^{2+} ion, while Cu^{2+} tolerant bacteria could adapt up to 20µM Cu^{2+} ions. The Ag^{+} tolerant bacteria have the lowest tolerance, which is only up to 5µM of Ag^{+} ion concentration. These isolates are of

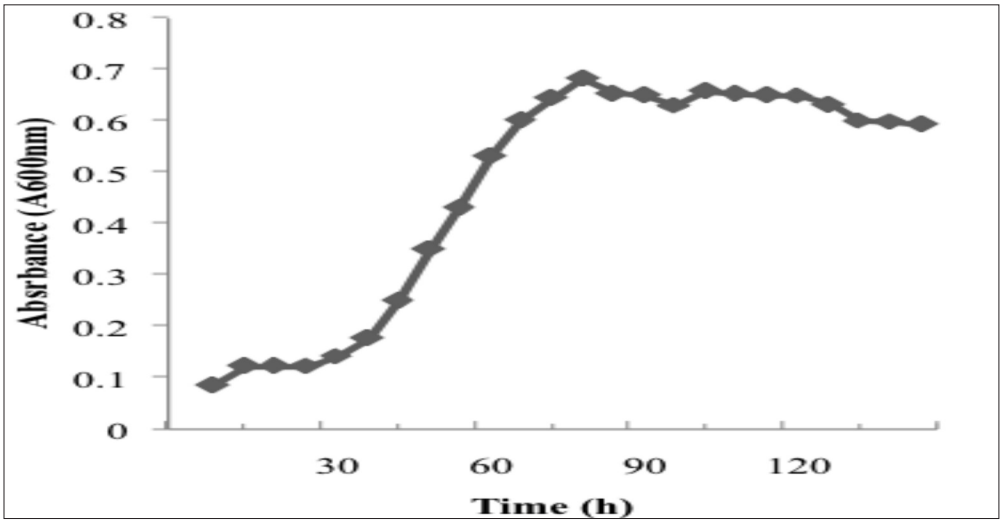

Fig.5: The growth curve of *Bacillus* sp in CFM liquid medium in the absence of metal ion

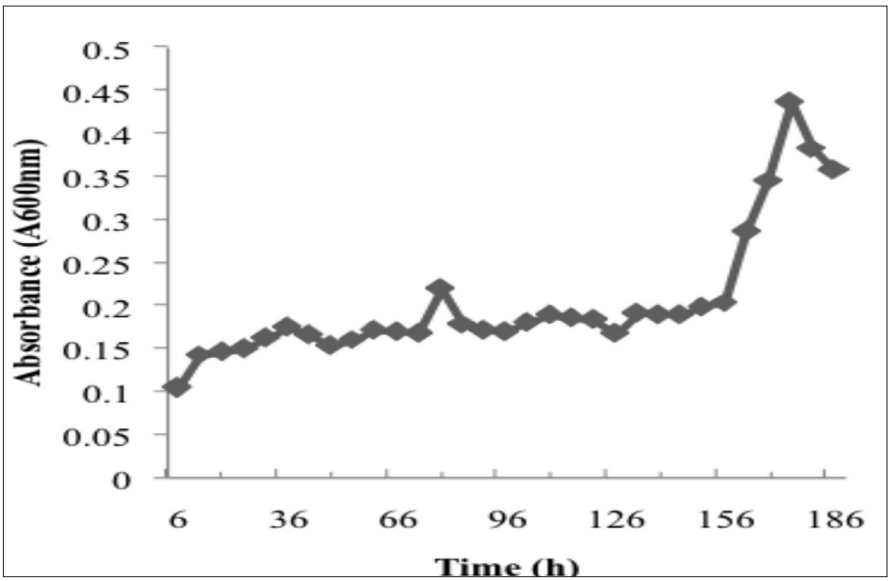

Fig.6: The growth curve of *Achromobacter* sp. in CFM liquid medium in the absence of metal ion

interest for further characterization in order to understand their mechanisms for metal tolerance and they have the potential to be developed for bioremediation of toxic heavy metals in contaminated environments.

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Cytotoxic Properties of Selected *Etlingera* spp. and *Zingiber* spp. (Zingiberaceae) Endemic to Borneo

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ABSTRACT

Zingiberaceae are known as valuable herbs with an important role in the prevention and treatment of various diseases. More than 300 species of Zingiberaceae were documented in Borneo. In this study, methanolic extracts of three species of Zingiberaceae (namely, *Etlingera velutina*, *Etlingera belalongensis* and *Zingiber vinosum*) were analysed for their total phenolic and flavonoid contents and cytotoxic activity *in vitro*. The cytotoxic activities of these extracts were tested against several cancer cell lines, such as hormone dependent breast cancer (MCF-7), non-hormone dependent cancer (MDA-MB-231), ovarian cancer ($CaOV_3$) and cervical cancer (Hela) using MTT assay. Crude extracts from rhizome of *E. belalongensis* and *E. velutina* showed significant cytotoxic activity against MDA-MB-231 cell line proliferation, with IC_{50} values (concentration which inhibit 50% of cell population) of 51.00±4.24 µg/ml and 67.00±9.89 µg/ml, respectively. The methanol extracts were further analysed for the cell cycle analysis using flow cytometry. The results showed that the *Etlingera* species exhibited higher antioxidant activity and stronger cytotoxic activity in selected cancer cell lines, with the highest cell death accumulated in G1 phase as compared to *Zingiber* species. Thus, polyphenol phytochemicals could be the major contributors to the cytotoxic activity of these species. As a conclusion, tropical gingers in Borneo investigated in this study have the potential to be developed as anticancer remedies.

Keywords: Zingiberaceae, *Etlingera* spp., *Zingiber* spp., total phenolic and flavonoid contents, cytotoxic

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INTRODUCTION

Cancer is one of the predominant killers in the world and it represents a real crisis for public health worldwide. According to the statistics by WHO in 2005, out of 7.6 million of deaths worldwide, about thirteen percent were caused by cancer (WHO, 2005) and the number increases by years (WHO, 2008). The use of medicinal plants as an alternative method to cure cancer has been established by WHO since 1978 (WHO, 1978). Several plant-derived compounds are currently successfully employed in cancer treatment with plant products as the main sources of drugs (Hernandez-Ceruelos *et al*., 2002).

Herbs, fruit and vegetables contain a variety of phytochemicals, including flavonoids which have antioxidant and anticancer properties. Of the estimated 250,000 – 500,000 plants species, only a small percentage has been investigated phytochemically and an even smaller percentage has been properly studied in terms of their pharmacological properties (Rates, 2001).

Zingiberaceae consist of 50 genera and 1,500 species worldwide and at least 20 genera and 228 species are found in Malaysia. Generally, Zingiberaceae are valuable herbs with an important role in the prevention and treatment of diseases (Wang & Duan, 1999) and an ingredient in more than half of all traditional Chinese medicines. Besides that, species from the family Zingiberaceae are often used in 'Jamu' (Indonesian traditional herbal medicine). In the present study, the cytotoxic activities of the methanolic extracts of the samples were tested against several cancer cell lines, such as hormone-dependent breast cancer (MCF-7), non-hormone-dependent breast cancer (MDA-MB-231), ovarian cancer ($CaOV_3$), and cervical cancer (Hela) by using MTT assay. In addition, the cell cycle analysis was also conducted to study whether these extracts could affect the cell cycle events. Zingiberaceae have been shown to display anticancer properties. Several Zingiberaceae samples, extracted with different solvents (i.e. petroleum ether, chloroform and ethanol), displayed strong anti-tumour activity (Vimala *et al*., 1999; Murakami *et al*., 1993). Murakami *et al.* (1994) reported that active constituents isolated from *Zingiber cassumunar* Roxd displayed a promising new anti-cancer drug. Besides that, Zerumbone which was extracted from *Zingiber zerumbet* also displayed a significant anticancer activity (Abdul *et al*., 2008). In another study, *Zingiber officinale* varieties (Halia Bara and Halia Bentong) were found contain seven important flavonoids and these compounds have been shown to display dominant anticancer activity (Ghasemzadeh *et al*., 2010).

MATERIAL AND METHODS

Plant Material and Sample Preparation

Fresh samples were collected from Tawau Hills Park and Crocker Range Park in Sabah. The herbarium voucher specimens were identified and deposited by Mr. Januarius Gobilik from Forest Research Centre, Sandakan, Sabah. All the plants were frozen at -20^{0}C and lyophilized for 48 h at 13.3 Pa

in freeze-dryer (Labconco, vacuum pump RV12, Edwards). After drying, the samples were ground and stored in air-tied plastic bags for further use.

Extraction

The freeze-dried samples were ground into fine powder. Fifty gram of the sample was extracted with 100 mL methanol for three days. The resulted slurry was vacuum-filtered through a Whatman No. 3 filter paper and the filtrate was subjected to vacuum rotary evaporation (Rotavapor model R110, Buchi, Flawil, Switzerland) at 40^{o}C. The concentrated methanolic extracts were stored in amber glass vials at 4^{0}C until used.

Determination of Total Phenolic Contents

Total phenolic content was determined using Folin-Ciocelteu, as described by Velioglu *et al*. (1998) with slight modification. Follin-ciocelteu reagent was diluted 10-folds with distilled water. Three hundred microliters of the extract was mixed with 2.25 ml of Folin-Ciocalteu reagent solution. The solution was mixed well using vortex and then allowed to stand for 5 min in the room temperature; 2.25 ml of the sodium bicarbonate (60 g/L) solution was added to the mixture. After 90 min in the room temperature, absorbance was measured at 725 nm using a spectrophotometer. Gallic acid was used as a standard. A standard concentration curve from 1 mg/ml to 5 mg/ml at 1 mg/ml interval was plotted. The total phenolic content of the extracts was determined from the standard graph. The results are expressed as mg gallic acid equivalent.

Determination of the Total Flavonoid Contents

The determination of the total flavonoids content was performed according to the colorimetric assay by Kim *et al*. (2003), with a slight modification. Distilled water (4ml) was added into the extracts (1 ml). Then, 5% sodium nitrite solution (0.3ml) was added, followed by 10% aluminium chloride solution (0.3ml). Test tubes were incubated at ambient temperature for 5 min, and this was followed by additions of 2 ml of 1 M sodium hydroxide and 2.1 ml of distilled water into the mixture after 6 minutes. The mixture was thoroughly vortexed and the absorbance of the pink colour developed was determined at 510 nm. The calibration curve was prepared with catechin and the results were expressed as mg catechin equivalents (CEQ)/100 g sample.

Cell Culture

MCF-7, MDA-MB-231, $CaOV_3$ and Hela cell lines were obtained from American Type Culture Collection (ATCC, USA). The RPMI 1640 Medium (Gibco, USA), supplemented with 10% of foetal calf serum (Gibco, USA) and 1% of penicillin streptomycin (Gibco, USA), was used to culture cell lines in 25 ml flasks (Nunc, Denmark), and incubated in 5% CO_2 incubator (Sanyo, Japan) at 37^{0}C.

MTT Assay for Cell Proliferation

The cytotoxic effects of the plant extracts against previously mentioned human cancer cell lines were determined by a rapid colorometric assay, using MTT bromide and compared with an untreated control (Mosmann, 1983). The concentration of MTT solution used was 5 mg/ml. The MTT solution was prepared by dissolving 0.05 g of the MTT powder in 10 ml PBS (pH 7.2). This solution was filtered through a 0.2 µm filter and covered with aluminum foil to avoid exposure to light. This solution was stored at 4^0C prior to use. Solubilisation buffer was prepared by dissolving 10% SDS (Sodium dodecyl sulphate) in PBS solution. The 96-well microtiter plates, containing cell culture solution, were removed from the incubator after 72 hour of incubation. 10 µL of 5 mg/ml MTT solution was added into each well, including the control wells. After adding the sample extracts, a new medium was added to make up the final volume of 100 µL in each well. The plate was incubated in a 5% CO_2 incubator (Sanyo, Japan) at 37°C for 72 h. Then, 20 µL of MTT reagent was added into each well. This plate was incubated again for 4 h in CO_2 incubator (Sanyo, Japan) at 37^0C. Subsequently, 100 µL of solubilization solution was added into each well to dissolve the remaining purple colour formazon crystals. The cell was then left overnight in 37^0C CO_2 incubator. Finally, the absorbance of the formazan was determined at 550 nm using an ELISA reader (LX-800). Meanwhile, Vincristine (anticancer compound) was used as a positive control.

Cell Cycle Analysis

The cell cycle was analyzed using flow cytometry (FCM) (Model Cyan ADP, Denmark) analysis (Yuan *et al*., 2004). A total of 2 x 10^5 cells were harvested from the control culture and the cells treated with the extracts after 72 hour of the incubation period. The cells were washed twice with PBS and fixed in 70% ethanol for 2 hours. The samples were then concentrated by removing ethanol. Cellular DNA was stained with 500µl of 10µg/ml propidium iodide in 100µg/ml of RNase for 30 minutes in the dark and in room temperature.

Statistical Analysis

All the experiments were carried out in 3 independent experiments and all the data were presented as a mean ± standard deviation of mean using SPSS version 15.0. The data were statistically analysed by one-way ANOVA and Duncan's test. A significant difference was considered at the level of $p < 0.05$.

RESULTS AND DISCUSSION

Many studies have been conducted to determine the contribution of phytochemicals by plants as antioxidants and anti-cancer agents. Plant extracts that are rich in polyphenols and other phytochemicals may contribute to antioxidant and anti-cancer activities. There are many types of phytochemicals including flavonoid, phenolic, steroid, terpenoids and alkaloid. Phenolic and flavonoid, which occur in plants, are very dominant phytochemicals (Manach, 2004) and these compounds

have the potential to benefit human health. In fact, these compounds have been shown as a group of chemicals that may possess antioxidant activity (Shahidi & Wanasundara, 1992) and have physiological functions that include anti-mutagenic and anticancer properties (Kono *et al*., 1995). This effect could be due to their redox properties (Zheng & Wang, 2001), which play important roles in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.

The total phenolics contents in the methanolic extract were in the range of 41.7 mg GAE/g to 5.3 mg GAE/g dry weight (dw). The rhizomes of *Z. vinosum* and *E. velutina* displayed the highest total phenolic content ($p<0.05$), while the lowest was shown by the stem of *E. velutina*. For all the species tested, the results showed that the phenolic contents were distributed more abundantly in the rhizome as compared to the stem (Table 1). Past studies have also shown that the antioxidant activities of the ginger species were concentrated in the rhizomes (Jitoe *et al*., 1992; Habsah *et al*., 2000). Meanwhile, the rhizome of gingers has been reported to contain antioxidant activity comparable to that of α-tocopherol (Zaeoung *et al*., 2005). The isolated compounds of zerumbone and kaempferol from *Z. aromaticum* showed a potent antioxidant activity (Usia *et al*., 2004). Similarly, Akiyama *et al*. (2006) also reported that diarylheptanoid isolated from *Z. ottensii* displayed a better scavenging activity as compared to L-ascorbic acid or α-tocopherol.

In this experiment, the concentration of flavonoids in the ginger extract was expressed as the mg of catechin equivalents per g of the extract (Table 1). For the total flavonoid content determination, the results showed the same trend with the total phenolic content. The total flavonoid in the

TABLE 1
Total phenolic and total flavonoid contents of methanolic extracts from selected *Etlingera* and *Zingiber* species

Samples	Part	% yield extract	Total phenolic (mg GAE/g)[a]	Total flavonoid (mg CE/g)[b]
E.belalongensis	rhizome	25.76	17.07 ± 0.32[b]	3.77 ± 0.15[a]
	stem	25.53	10.07 ± 0.25[b]	2.57 ± 0.15[a]
E.velutina	rhizome	30.31	25.03 ± 0.46[c]	7.63 ± 0.06[a]
	stem	29.18	5.30 ± 0.10[a]	2.80 ± 0.20[a]
Z.vinosum	rhizome	35.63	41.70 ± 1.11[d]	8.50 ± 0.20[a]
	stem	27.97	27.97 ± 0.93[c]	3.37 ± 0.06[a]

Total phenolic content was expressed as mg gallic acid equivalent in 1 g of dry sample. Values are presented in mean ± S.D (n=3); those with different letters are significantly different at $p < 0.05$, as measured by Tukey HSD test. ANOVA compares the values between rhizomes and stems of each species.
[a] Total phenolic was expressed as gallic acid equivalent (GAE) in 1 g of dry sample.
[b] Total flavonoid was expressed as catechin equivalent (CE) in 1 g of dry sample.
E = Etlingera; Z = Zingiber.

methanolic extracts was in the range of 8.5 mg GAE/g to 1.97 mg GAE/g dry weight (dw). The rhizome of *Z. vinosum* displayed the highest total flavonoid content ($p<0.05$), while the stem of *E. velutina* showed the lowest. From the previous study, the flavonoid compounds in Zingiberaceae have been reported to possess strong antioxidant properties (Cai *et al*., 2006), while the major component of the essential oil extracted from *Z. zerumbet* showed a more promising use as anti-inflammatory and chemotherapeutic agents (Tanaka *et al*., 2001). Masuda *et al.* (1991) reported the occurrence of several sesquiterpenoid and flavonoid in the rhizome of *Z. zerumbet*.

As for the cytotoxicity study, each sample was screened for cytotoxicity against several cancer cell lines, such as MDA-MB-231, MCF-7, CaOV3 and Hela using MTT assays. The cell killing and inhibition of proliferation could be explained by the reduction in the number of cells by particular agent (extract). The results showed that the sample extracts displayed a cytotoxic activity against MDA-MB-231 cancer cell line, with IC_{50} (i.e. the concentration that inhibits 50% of cell lines) ranging from 51 µg/ml to 96 µg/ml after 72 hours of treatment (Table 2). The possible mechanism of the cytotoxic activity of the plant extracts was further investigated using cell cycle analysis through flow cytometry. The results showed that all the extracts arrested cancer cells in sub-G1 phase (Table 3). The results for the samples showed the same trend with a positive control (Vincristine) and displayed significant differences as compared to the control ($p<0.05$).

Numerous cell cycle analyses have proven that good anti-cancer drugs arrested the cell in sub-G1 phase. In agreement to this, Kim (2005) reported that [6]-gingerol isolated from *Z.officinale* inhibited an angiogenesis of human endothelial cells and caused the cell to arrest in the sub-G1 phase. This result was also supported by Choi and Kim (2008) who had shown that daidzein (flavonoid) caused cells to arrest

TABLE 2
IC_{50} values of the methanol extracts of selected *Etlingera* and *Zingiber* species against MDA-MB-231, MCF-7 and CaOV3 cell line

Samples	Plant Part	MDA-MB-231	MCF-7	CaOV3	Hela
E. belalongensis	Rhizome	51.00±4.24^{c}	>100	>100	>100
	Stem	74.00±2.83^{c}	>100	>100	>100
E. velutina	Rhizome	67.00±9.89^{c}	>100	>100	>100
	Stem	89.50±14.85^{d}	>100	>100	>100
Z. vinosum	Rhizome	89.00±7.78^{d}	>100	>100	>100
	Stem	96.00±2.83^{d}	>100	>100	>100
Vincristine		13.00±3.14^{b}	8.50±3.41^{a}	17.50 ±0.82^{b}	3.00±0.73^{a}

E = Etlingera; Z = Zingiber
Values are expressed as mean ± standard deviation (n=3), in which those with different letters are significantly different at $p<0.05$

TABLE 3
Cell cycle analysis of MDA-MB-231 treated with methanol extracts from selected *Etlingera* and *Zingiber* species for 72 hours

Samples	Phase	Control	Treatment
**E. belalongensis* (stem)	sub-G1	5.72±0.18[a]	**68.72±2.03[c]**
	G0/G1	80.22±0.21[d]	25.67±1.75[b]
	S	2.75±0.30[a]	2.46±0.33[a]
	G2/M	11.61±0.16[b]	3.46±0.16[a]
**E.velutina* (rhizome)	sub-G1	5.45±0.07[a]	**83.44±0.63[d]**
	G0/G1	80.3±0.33[d]	13.57±0.48[b]
	S	2.68±0.46[a]	1.86±0.23[a]
	G2/M	11.57±0.16[b]	1.36±0.06[a]
**Z.vinosum* (rhizome)	sub-G1	5.72±0.61[a]	**56.86±1.11[c]**
	G0/G1	76.49±0.58[d]	38.39±0.59[b]
	S	2.75±0.42[a]	1.71±0.02[a]
	G2/M	11.61±0.52[b]	3.15±0.33[a]
Vincristine	sub-G1	5.79±0.23[a]	**90.07±0.43[d]**
	G0/G1	79.79±0.07[d]	5.74±0.26[a]
	S	2.73±0.31[a]	2.07±0.18[a]
	G2/M	11.69±0.19[b]	2.12±0.24[a]

Values are presented in mean ± S.D (n=3); those with different letters are significantly different at $p < 0.05$, as measured by Tukey HSD test.
*Concentration of sample = 80 µg/ml.
Concentration of positive control = 5µg/ml.

in G1 phase in the human breast cancer cells. In addition, green tea polyphenol has been shown to suppress the proliferation of MDA-MB-231 and accumulated the cell at G1 phase (Thangapazham *et al*., 2006). Polyphenol that presents in the species may contribute to the cytotoxicity activities in the cancer cell lines. In conclusion, the methanolic extracts of selected *Etlingera* and *Zingiber* species endemic to Borneo have a great potential to be developed as an anti-cancer agent and are applicable to food and herbal products.

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Development of Multifunctional Biofertilizer Formulation from Indigenous Microorganisms and Evaluation of Their N_2-Fixing Capabilities on Chinese Cabbage Using ^{15}N Tracer Technique

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ABSTRACT

Biofertilizer is an alternative to chemical fertilizers to increase soil fertility and crop production in sustainable farming. Most biofertilizer products consist of a single function micro-organism such as N_2 fixing bacteria. This paper discusses the development of multifunctional biofertilizer products, based on indigenous micro-organisms that have all the desired characteristics, including plant growth promoting, phosphate solubilising and antagonistic towards pathogens, and optimisation of the micro-organisms present in the modified "Natural Farming" compost. Composting through the "Natural Farming" method is a simple and cheap method to turn empty fruit bunches (EFB) of oil palm into compost. Indigenous micro-organisms in each stage of composting were isolated and screened for the abilities to solubilise phosphate and produce indole-3-acetic acid (IAA). These indigenous micro-organisms were developed into biofertilizer products. Effects of these products on plant growth of Chinese cabbage and contribution of N_2 to the plants were evaluated using the ^{15}N isotopic tracer technique in a greenhouse trial. Fertilizer treatment using a combination of microbial strains (T7) was found to significantly enhance the growth of Chinese cabbage. All the plants receiving biofertilizer microorganisms showed N_2-fixing effects as compared to the control (T9). The isolated indigenous micro-organisms may enhance plant growth through N_2 fixation, solubilising insoluble inorganic phosphate compounds or hydrolyse organic phosphate to inorganic P or stimulation of plant growth through hormonal action such as produce IAA. Combination of microbial strains could be a good multifunctional biofertilizer for sustainable agriculture.

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INTRODUCTION

Responding to global warming and global challenges in crop production, Malaysia is steadily adopting sustainable agriculture. Agro-waste management and enhancement of biodiversity are the approaches towards sustainability (Shukor, 2009; Ong, 2009). Empty fruit bunches (EFB) of oil palm are one of the agricultural wastes that are building up at alarming rates at palm oil factories in Malaysia. This particular material is difficult to manage if not treated or turned into valuable products like compost. Meanwhile, micro-organisms are an important component of world biodiversity (Sadi *et al*., 2006). These micro-organisms include phosphate solubilisers, plant growth promoters and nitrogen fixing bacteria (Umi Kalsom & Sariah, 2006). Composting through the modified "Natural Farming" method is simple and cheap at turning EFB into compost. Moreover, it is a natural agro-management method, utilising agricultural waste and indigenous soil micro-organisms. This method was developed in Korea (Cho & Kayoma, 1997), and has been gaining acceptance in several countries and in Malaysia, by the Department of Agriculture. This method involves five stages of composting processes producing inoculants of indigenous microorganisms (IMO). Biofertilizer is a substance containing living micro-organisms, which are applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant, and promote growth by increasing the supply or the availability of primary nutrients to host plants (Vessey, 2003). The objectives of the present study were to isolate and utilise the indigenous micro-organisms from each stage of the composting process to produce bacterial isolates that could be developed as multifunctional biofertilizer micro-organisms. The abilities of the indigenous micro-organisms to solubilise phosphate and produce indole-3-acetic acid (IAA) were evaluated. The potential micro-organisms were selected for development of biofertilizer. The micro-organisms were further evaluated on their ability to fix N_2. The effects of these products on the plant growth of Chinese cabbage and the contribution of N_2 using the ^{15}N isotopic tracer technique in a greenhouse trial were particularly studied.

MATERIALS AND METHODS

IMO Preparation

Composting of EFB, through the modified "Natural Farming" method (Wahid, 2005; Cho and Kayoma, 1997), was conducted as follows: EFB was dried, ground and mixed with composting agents - rice, bran and sugarcane molasses through five stages of composting processes producing inoculates of indigenous microorganisms (IMO). Rice was packed and fermented for 2 days (IMO 1). IMO 1 was then mixed with sugarcane molasses and fermented for a week (IMO 2). IMO 2 was mixed with 1 L of water and 8 kg bran, and incubated for 5 days (IMO 3). After 5 days, IMO 3 was mixed with soil and incubated for 5 days (IMO4). Finally, 200 kg griddled EFB (60% moisture content) was mixed with IMO 4 and fermented for 2 weeks. In each stage, indigenous micro-

organisms were isolated by using ten-fold serial dilution technique.

IAA Production

The isolates were tested for indole-3-acetic acid (IAA) production by culturing on TSA amended with 1-tryptophan, followed by overlying them with 82 mm diameter nitrate cellulose membrane and incubating at 28 ˚C for 3 days. The membranes were overlaid on a filter paper saturated with Salkowsky's reagent (Gordon & Webber, 1950; Alvarez *et al*., 1995). Isolates producing IAA showed pink to red colour after 0.5 to 3 hours. The isolates were also tested for their ability to solubilise phosphate.

Phosphate Solubilising Test

In the phosphate solubilising test, the isolates were cultured on phosphate agar plate (Freitas *et al.*, 1997) and incubated for 14 days. The isolates which produced clear zones were selected and developed into biofertilizer products.

Greenhouse Study of Biofertilizer for N_2-fixing Capabilities and Plant Growth

Biofertilizers were prepared by culturing three selected isolates viz. AP1, AP2 and AP3 on tryptic soy broth for 24 hours. These isolates were individually mixed and in combination with the sterile carrier irradiated by gamma process (Phua *et al*., 2009). The effectiveness study of these products on the growth of Chinese cabbages was carried out in the greenhouse (Table 1). The N_2-fixing activity assessment was carried out using ^{15}N dilution method. A week before transplanting, 0.1 g of ^{15}N labelled ammonium sulphate (10.18 % atom excess) was mixed with 1 kg of soil (FNCA, 2006). Two-week-old seedlings were transplanted into pots containing 1 kg of soil mixture containing soil, peat and sand in the ratio of 2:1:1. Crops were harvested after two months, and their dry weights were also determined. The abundance of ^{15}N in the samples was determined by emission spectrometry after Kjeldahl digestion and titration of digests. The percentages of N derived from labelled fertilizer (%Ndff), atmosphere (%Ndfa) and soil (%Ndfs) were calculated by using the following equations:

$$\% \text{ Ndff} = \{^{15}\text{ nae}/^{15}\text{N (10.18)}\} \times 100\ \%$$

$$\% \text{ Ndfa} = \{1 - (\text{ndff treatment}/\text{ndff control})\} \times 100\%$$

$$\% \text{ Ndfs} = 100 - \% \text{ ndff} - \% \text{ ndfa}$$

Data were analyzed by ANOVA, with the means separated by Duncan's test ($P \leq 0.05$).

TABLE 1
Treatments for greenhouse experiment

Treatments	
T1	AP1
T2	AP2
T3	AP3
T4	AP1 + AP2
T5	AP1+AP3
T6	AP2+AP3
T7	AP1+AP2+AP3
T8	NF
T9	Control

Key:
AP1 = Phosphate solubilise and antagonistic micro-organisms against Bacterial Wilt
AP2 = Plant growth promoter and phosphate solubilise
AP3 = Phosphate solubilise
NF = Natural Farming Compost
Control = Receiving ^{15}N only

RESULTS AND DISCUSSION

"Natural Farming" composting method is a simple and cheap method for the production of EFB compost. The compost became matured within one and a half months, while other composting methods take three to four months. A total of 56 indigenous micro-organisms were isolated from the five stages of IMO. There were 8, 11, 13, 13 and 11 indigenous micro-organisms isolated from IMO 1, IMO 2, IMO 3, IMO 4 and IMO 5, respectively. Sixteen of the bacterial isolates were Gram positive and others were Gram negative. The result derived from the IAA test showed that two isolates were IAA producers and six isolates produced clear zones on phosphate agar plates indicating phosphate solubilising activity. These isolates had been developed into biofertilizers.

The greenhouse experiment for the evaluation of the effect of biofertilizers on plant growth showed that all the treated plants significantly increased ($p < 0.05$) the dry weights of the test plants compared to the control (T9). Meanwhile, the treatment using a combination of microbial strains (T7) was found to have significantly enhanced the growth of Chinese cabbage (Fig.1). The combination treatments showed better results as compared to the single treatments. Han *et al.* (2006) also showed that the combined treatment of *Bacillus megaterium* var. *phosphaticum* and *Bacillus mucilaginosus* increased the availability phosphorus and potassium in soil, and thus, increasing the uptake and plant growth of pepper and cucumber. Sarma *et al*. (2009) reported a combination bio-inoculation, namely, two *Fluorescent pseudomonas* strains, increased *Vigna mungo* yield by 300% in comparison to the control crop. These results indicated that a combination of beneficial micro-organisms might increase the nutritional assimilation of plant and total N in soil.

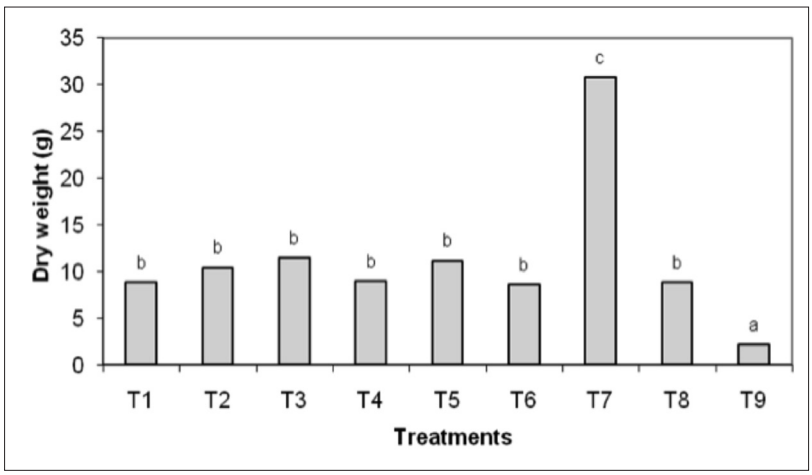
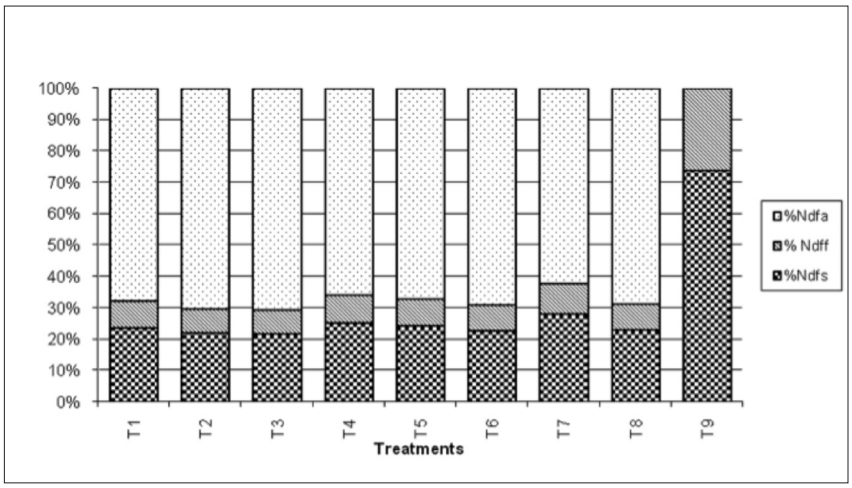


Fig.1: Dry weights (g) of Chinese Cabbage



Keys: % Ndfs = % N derived from the soil
% Ndff = % N derived from the labelled fertilizer
% Ndfa = % N derived from the atmosphere

Fig.2: Effects of biofertilizer treatments in contributing N from N_2 to Chinese cabbage plants by using ^{15}N isotopic tracer

Fig.2 illustrates that all the treated plants have the N_2-fixing effects as compared to the control (T9). It seems probable that there is an influence of these phosphate solibilising bacteria on N uptake. Previous reports have shown the influences of phosphate solubilising micro-organisms on nitrogen uptake and root zone biodiversity. Linu *et al*. (2009) showed that phosphate solubilise improved nodulation, root and shoot biomass, straw and grain yield and phosphorus and nitrogen uptake of cowpea. Similarly, Kuey *et al.* (1989) also reported phosphate solubilise helped increase the ability of accumulated phosphate, efficiency of biological nitrogen fixation and increase the availability of Fe, Zn etc., through production of plant growth promoting substances. Therefore, the isolated indigenous microorganisms may enhance the plant growth through N_2 fixation, solubilising insoluble inorganic phosphate compounds, hydrolyse organic phosphate to inorganic P or stimulation of plant growth through hormonal action such as produce IAA. Combination of microbial strains could be a good multifunctional biofertilizer for sustainable agriculture.

CONCLUSION

The modified "Natural Farming" composting method is a simple, cheap and fast method used to produce EFB compost that contains beneficial micro-organisms with the potential to be developed into biofertilizer. Meanwhile, multifunctional biofertilizer products produced from the combination of indigenous micro-organisms have been shown to enhance the growth of Chinese cabbage and contribute N through fixation

of atmospheric N_2 by the micro-organisms in a greenhouse trial.

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How Valuable is Degraded Habitat to Forest Birds? A Case Study in Bachok, Kelantan

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ABSTRACT

Conservationists usually pay less attention to degraded habitats than primary forests since the former areas generally support less number of species. In this study, diversity and abundance of birds inhabiting degraded habitats were recorded in order to assess the capability of these habitats in conserving birds, particularly forest species. For this purpose, five visits were done to the district of Bachok, Kelantan, Malaysia, from June 2008 until May 2009. The study area comprised mainly of small villages intersperse with small trees or shrubs and cash crop areas. A direct observation method was used to record the bird diversity in the study area. A total of 70 bird species were recorded in the study area and most of them are residents and insectivores, indicating that insects are abundant in the study area. In term of habitat utilisation, most recorded species are usually associated with open and country habitats, mangroves, as well as garden and parks. A total of nine species or 13% of the birds recorded in this area have greater association with forest habitats. Some of these forest birds were observed feeding while others having their nests in the area. Although degraded habitat in Bachok area can play important roles in conserving forest birds, the value of these habitats cannot be established since these birds are not exclusively forest dependent and can be commonly found in secondary or disturbed forests. Therefore, further studies on the behavioural aspects of forest birds need to be carried out to determine the level of resources required by forest specialists in degraded habitat.

Keywords: Forest birds, forest disturbance, habitat displacement, bird survival, Peninsular Malaysia

INTRODUCTION

Most studies on tropical birds' diversity have been conducted in primary forests, and this is possibly because many resident species (at least 70%) in this region are partly or exclusively dependent upon this habitat.

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Furthermore, most human-modified areas in the tropics have largely been considered hostile to biodiversity. Consequently, only a few conservation initiatives have focused on secondary forests, agro-forestry, or other human-modified areas. On the contrary, the recent findings suggest that degraded habitats or secondary forests have high conservation potential (Sodhi *et al*., 2005; Sekercioglu *et al*., 2007; Edwards *et al*., 2010), and therefore, demanding more studies to be conducted beyond primary forest for a better preserved biodiversity (Gardner *et al*., 2009). This is because 90% of the world's tropical forests exist outside of the primary forest and 60% of the world's remaining tropical forests are either degraded or secondary forests (Schmitt *et al*., 2009). Globally, it is reported that 42 tropical countries have more secondary forests or degraded habitats as forest covers than primary forests (FAO, 2009). In Malaysia, only 18.3% of its forests were covered by primary forests (out of 20.89 million hectares of forested area) and this figure keeps on deteriorating as deforestation rate is accelerating. In other South–East Asian countries, the remaining primary vegetation varies from 3% in the Philippines, 5% in Indo-Burma, 8% in Sundaland and 15% in Wallacea (FAO 2009).

Therefore, the fate of many species is depending on what happens to the other habitats outside the primary forests. Among the habitats that require further attention are secondary forest, agricultural areas, rural or human settlements areas, and other human-modified landscapes. Several studies have been conducted to assess the capability of degraded habitat in conserving forest birds (e.g., Wong 1986; Zakaria *et al*., 2002; Peh *et al*., 2005, 2006; Sodhi *et al*., 2005; Barlow *et al*., 2007; Sekercioglu *et al*., 2007; Edwards *et al*., 2010). The results indicate that degraded habitats can, in some cases, serve as surrogate habitats for some of the forest birds.

Meanwhile, patterns of habitat use and occupancy suggest that degraded habitat in the region (which is primarily abandoned pasture) may only be valuable to forest birds after a specific level of regeneration and during certain times of the year. Therefore, degraded landscapes could act as good refuges for the forest birds if it were allowed to regenerate. Forest birds are more sensitive to disturbance because their survival depends on the availability of forest's resources (Sodhi, 2002; Sodhi *et al*., 2005; Sekercioglu *et al*., 2007; Zakaria & Zamri, 2008; Ramli *et al*., 2010). Among the required resources are food and water, suitable nesting sites and nest materials, lack of predators and competitors, as well as suitable mating partners. Theoretically, any disturbed habitat will be able to harbour forest birds if they can supply these resources. For instance, a good proportion of the forest birds are able to survive in disturbed habitat in the southern part of Johor (Peh *et al*., 2005).

Although a moderate number of biodiversity studies have been conducted on secondary forests, the least research was carried out in other types of degraded

habitats, such as agricultural area or other human-modified landscapes. This is despite the recent interest in the diversity patterns and conservation strategies for the native species in agricultural area and human-modified landscapes due to the current global changes in land use. It is not known how valuable the agricultural lands and other rural human-dominated landscapes for biodiversity conservation, especially to forest birds. Therefore, this study was designed to assess the significance of degraded habitats (human settlement, cash-crop, and shrubs) in conserving forest birds. To achieve this objective, the abundance and species richness of the birds inhabiting degraded habitats were recorded.

MATERIALS AND METHODS

The study was conducted in the rural area of the district of Bachok in Kelantan. The area is dominated by traditional villages and other human settlements which intersperse with cash-crop areas. There is no forested area within the district but the adjacent district (Pasir Puteh which is located approximately 5 km away) has few fragmented forest reserves (Ramli *et al*., 2010). Eight study sites (identified as site A to site H) with different physical characteristics were established within the study area (Table 1). Among the habitats available in study area are mangroves, open grazing fields (some with electrical pylon and cables), and shrubs (which consist of small and large trees). A total of five visits (comprised three days each) were conducted to the study area

TABLE 1
Location of the study sites and their descriptions

Site	Site Descriptions	Latitude	Longitude
SITE A	Shrubby areas with small trees including small patch of mangrove forest, and some open grazing fields.	N 05° 57.433’	E 102° 26.628’
SITE B	Shrubby areas with small section of mangrove forest, is close to the beach and with some open grazing fields.	N 05° 57.499’	E 102° 26.277’
SITE C	Few tall trees with sparse shrubs, electrical cables and pylons.	N 05° 59.247’	E 102° 26.058’
SITE D	An open area with few tall trees, natural well and grazing fields.	N 05° 59.434’	E 102° 25.537’
SITE E	An open/grassy area with electrical pylon, and the absence of taller trees.	N 05° 59.136’	E 102° 25.422’
SITE F	A swampy area with freshwater supply in small stream and concrete drain.	N 05° 59.390’	E 102° 25.306’
SITE G	An open/grassy area, with few small trees and open grazing fields.	N 05° 59.661’	E 102° 25.188’
SITE H	An open area with a lot of coconut trees, and is adjacent to the beach.	N 06° 00.417’	E 102° 25.583’

from June 2008 to May 2009. The direct observation method (using binoculars of 8 X 40 magnifications) was used to record the bird species diversity at the area. Morning observation session started at 0730 hours until 1200 hours, whereas the afternoon observation session began at 1400 hours until 1830 hours. Each site was visited for 30 minutes before moving to the next site. Three-point count stations were established within each site and each point count lasted for 10 minutes (Sodhi *et al*., 2005; Lee & Marsden, 2008; Ramli *et al*., 2010). Any bird seen or heard within 50 metres radius was recorded. Doubtful sightings were confirmed by repeating the observations involving note-taking and drawings, which were later identified using standard field guides (such as those by Jeyarasingam & Pearson, 1999). Each site was visited twice daily (one each in the morning and afternoon sessions) and a proper schedule was established to ensure that all the sites were visited at different times. All the observed birds were identified up to the species level and secondary information related to each species (including habitat association and feeding guilds) was extracted from Jeyarasingam and Pearson (1999), Zakaria *et al*. (2002) as well as Zakaria and Zamri (2008). In this study, we concurred with Sodhi *et al*. (2005) in defining forest birds, i.e. those that occur mainly in lowland or low-montane forest habitats and used information provided by Jeyarajasingam and Pearson (1999), Zakaria *et al*. (2002), as well as Zakaria and Zamri (2008) in determining the association between the birds and their habitat.

RESULTS AND DISCUSSION

A total of 70 species of birds were recorded in the study area (Table 2). As for the resident species dominating the area (45 species or 64%), there are a few representatives of the introduced birds (three species), while migratory birds and birds that have both migrant and resident populations are represented by 11 species each (16%). Fifty two (52) species recorded in the study area could be commonly found throughout Peninsular Malaysia, whereas ten species were uncommon, and seven species were abundantly distributed. Interestingly, one of Malaysia's rare species, i.e. Javan Pond-Heron (*Ardeola speciosa*), was also recorded in the area. However, the local distribution pattern for some recorded species is different from that of the national distribution. Some species that are abundant or commonly found throughout Peninsular Malaysia, such as Eurasian Tree Sparrow (*Passer montanus*) and White-breasted Waterhen (*Amaurornis phoenicurus*), are uncommon or rarely found in study area. This difference is mainly due to availability of resources in the study area (Sodhi, 2002; Sodhi *et al*., 2005).

The presence of forest birds in the study area demands further explanation. Nine species (or 13%) of the forest birds were recorded in Bachok area. These are Chestnut-breasted Malkoha (*Zanclostomus curvirostris*), Greater racket-tailed Drongo

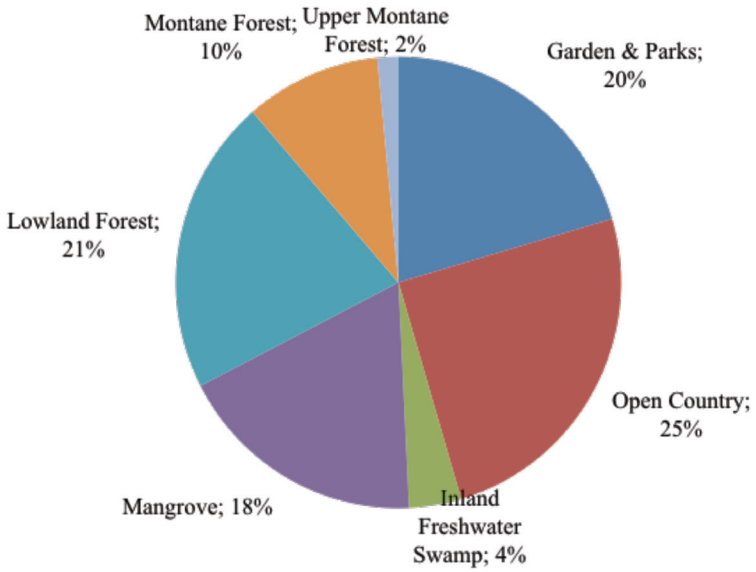

Fig.1: Composition of the bird species recorded in the study area according to their habitats

(*Dicrurus paradiseus*), Green-billed Malkoha (*Rhopodytes tristis*), Grey-breasted Babbler (*Malacopteron albogulare*), Rufous Woodpecker (*Micropternus brachyurus*), Rufous-fronted Babbler (*Stachyridopsis rufifrons*), Stripe-throated Bulbul (*Pycnonotus finlaysoni*), Tiger Shrike (*Lanius tigrinus*), and White-bellied Munia (*Lonchura leucogastra*). All the species are residents (except for Tiger Shrike) and fully protected by the Malaysian law (except for Chestnut-Breasted Malkoha and White-bellied Munia), and also commonly found throughout Peninsular Malaysia (except both species of babblers and White-bellied Munia which are uncommon). However, only Green-billed Malkoha, Chestnut-breasted Malkoha, and Rufous Woodpecker were frequently observed in the study area.

Among all the stations, only station E did not record any forest birds. The station is an open area with electrical pylon. Although it provides a suitable vantage point for carnivores of open area or parks, the station does not have much resource for forest birds. More forest birds were recorded at station H (5 species), which has coconut plantation, river mouth and shrubs. These kinds of habitats attract many insects which will draw insectivorous birds into the area. Other stations that managed to attract forest birds usually have shrubs, freshwater supply (such as small stream), and are close to the beach. This particular result is unfortunately predictable since most recorded species are birds that associate with open country, garden and parks, mangrove and lowland forests (Fig.1). It is understood that the composition of birds that associate with the first three habitats was recorded in higher number since the particular types of habitat are widely available in the study area.

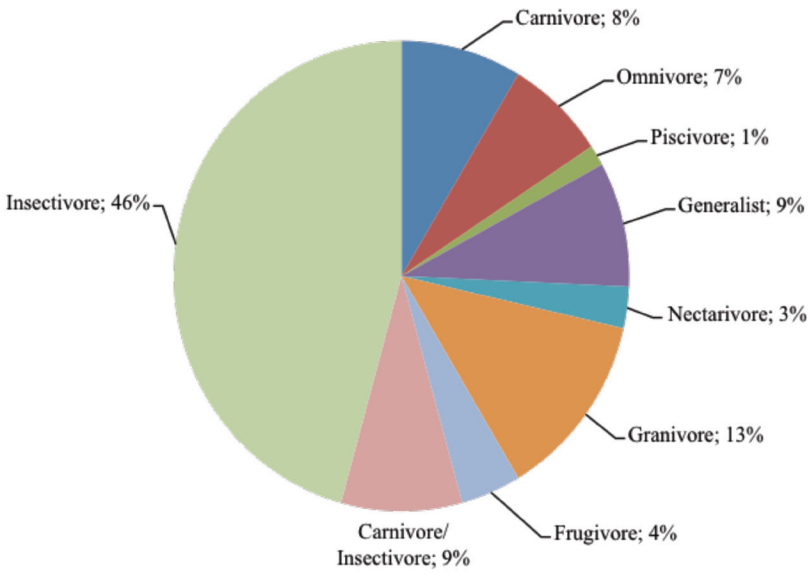

Fig.2: Composition of the bird species recorded in the study area according to their feeding guilds

Meanwhile, the availability of resources in each station plays important roles in attracting different bird species, especially forest birds. Sodhi (2002) postulated that frugivorous and insectivorous birds are more vulnerable to extinction after forest disturbance due to the decline in food supply. However, most forest birds that are able to survive in Bachok area are insectivores (32 species or 46%), while other feeding guilds are represented by less than nine species (Fig.2). Only Tiger Shrikes are carnivores, while Green-billed Malkoha predates invertebrates from understory foliage. Most forest birds recorded in the study area utilised the available degraded habitats as their resting and feeding sites but three species (namely, Chestnut-Breasted Malkoha, Green-billed Malkoha, and Stripe-throated Bulbul) were observed to be involved in breeding activities (i.e. they were either recorded close to their nests or bringing nesting materials).

In addition to the general behaviour of the species, higher frequency of detection (most of these forest birds were recorded more than once, either at various sites, or at different times) eliminated the possibility of misidentification or coincidence. For instance, Tiger Shrike was observed at three stations (C, F, and G), even during a single visit. This species had likely used the study area as the stop-over site during its migratory journey. On the other hand, two other forest species were recorded twice but at the same station. For example, Stripe throated Bulbul was recorded twice at station B (4-8 February, 2009 and 12-15 May, 2009), whereas Greater-racket tailed Drongo was observed twice at station H

TABLE 2
List of the species recorded in Bachok, Kelantan.

No.	Species	Distribution Status	Protection Status	Degree of Occurrence	Habitat Associations	Study Sites	Feeding Guilds
1	Ashy Minivet *Pericrocotus divaricatus*	M	TP	C	GP,OC,MG,LF	F	Insectivore
2	Ashy Tailorbird *Orthotomus ruficeps*	R	TP	C	MG,LF	A,B,	Granivore / Insectivore
3	Asian/Common Koel *Eudynamys scolopaceus*	R,M	TP	C	OC,MG	ALL	Frugivore / Carnivore
4	Banded Woodpecker *Chrysophlegma mineaceus*	R	TP	C	MG,LF, LMF	B,H	Insectivore
5	Barred Buttonquail *Turnix suscitator*	M	GB	C	OC	B,G	Granivore
6	Black Drongo *Dicrurus macrocercus*	M	TP	C	OC	E	Carnivore
7	Black-naped Oriole *Oriolus chinensis*	R,M	TP	C	GP,OC	A,B,D,G,H	Frugivore / Insectivore
8	Blue-tailed Bee-eater *Merops philippinus*	R,M	NP	C	OC	A,B,F	Insectivore
9	Blue-throated bee eater *Merops viridis*	R,M	TP	C	OC	A,F,G	Insectivore
10	Brahminy Kite *Haliastur indus*	R	TP	A	MG	C,E,F,G,H	Carnivore
11	Brown Shrike *Lanius cristatus*	M	TP	C	GP,OC	F	Carnivore / Insectivore
12	Brown-throated Sunbird *Anthreptes malacensis*	R	TP	C	GP,OC,MG	A,B,H	Insectivore / Nectarivore
13	Chestnut-breasted Malkoha *Zanclostomus curvirostris*	R	NP	C	LF	A,B,F,G,H	Carnivore / Frugivore

Table 2 (*continued*)

14	Chestnut-headed Bee-eater *Merops leschenaulti*	R	TP	UC	OC	A,C,F	Insectivore
15	Chinese Pond-Heron *Ardeola bacchus*	M	TP	C	IS,MG	A	Pisicivore / Invertebrate
16	Cinnamon Bittern *Ixobrychus cinnamomeus*	R,M	TP	C	IS	F,G	Pisicivore / Invertebrate
17	Collared Kingfisher *Todiramphus chloris*	R,M	TP	C	MG	B,E,H	Pisicivore
18	Common Flameback *Dinopium javanense*	R	TP	C	GP,OC,MG,LF	H	Insectivore
19	Common Iora *Aegithina tiphia*	R	TP	C	GP,OC,MG	A,F	Insectivore
20	Common Kingfisher *Alcedo atthis*	R,M	TP	C	IS,MG	H	Pisicivore
21	Common Myna *Acridotheres tristis*	R	NP	A	GP,OC	ALL	Frugivore / Insectivore
22	Common Tailorbird *Orthotomus sutorius*	R	TP	C	GP,OC,MG,LF,LMF	A,B	Frugivore / Nectarivore
23	Crested Serpent-Eagle *Spilornis cheela*	R	TP	C	MG,LF,LMF,UMF	B	Carnivore
24	Crimson breasted Flowerpecker *Dicaeum percussus*	R	TP	C	MG,LF	A	Nectarivore
25	Crow-billed Drongo *Dicrurus annectans*	M	TP	UC	MG,LF	E,F	Insectivore / Carnivore
26	Dark-necked Tailorbird *Orthotomus atrogularis*	R	TP	C	GP,MG,LF,LMF	B	Insectivore
27	Dollarbird *Eurystomus orientalis*	R,M	TP	C	OC	C,F,H	Carnivore / Insectivore

Table 2 (*continued*)

28	Eastern Cattle Egret *Bubulcus coromandus*	R,M	TP	C	OC	E,F,G	Pisicivore / Invertebrate
29	Eurasian Tree Sparrow *Passer montanus*	R	NP	A	GP,OC	B,H	Granivore
30	Glossy Swiftlet *Collocalia esculenta*	R	TP	C	OC,LF,LMF,UMF	ALL	Insectivore
31	Greater Coucal *Centropus sinensis*	R	TP	C	OC,LF	A	Carnivore
32	Greater Flameback *Chrysocolaptes lucidus*	R	TP	C	MG	H	Insectivore / Frugivore
33	Greater Racket-Tailed Drongo *Dicrurus paradiseus*	R	TP	C	LF,LMF	H	Insectivore / Carnivore
34	Green-billed Malkoha *Rhopodytes tristis*	R	TP	C	LF,LMF	A,B,C,D,F,H	Frugivore / Carnivore
35	Grey-breasted Babbler *Malacopteron albogulare*	R	TP	UC	LF	B	Insectivore / Granivore
36	Grey-faced Buzzard *Butastur indicus*	M	TP	C	OC	F	Carnivores
37	House Crow *Corvus splendens*	I	NP	A	GP,OC	A,B,C,E,H	Carnivores /Omnivores
38	House Swallow *Hirundo tahitica*	R	TP	C	OC	ALL	Insectivores
39	House Swift *Apus affinis*	R	TP	C	GP,OC,LF,LMF	ALL	Insectivores
40	Indian Roller *Coracias benghalensis*	R	TP	UC	OC	D,E,G	Insectivores
41	Japanese Sparrowhawk *Accipiter gularis*	M	OPB	C	OC,MG,LF	F	Carnivore
42	Javan Munia *Lonchura leucogastroides*	I	NP	C	GP,OC	G	Granivore

Table 2 (*continued*)

43	Javan Myna *Acridotheres javanicus*	I	NP	A	GP,OC	ALL	Frugivore / Insectivore
44	Javan Pond-Heron *Ardeola speciosa*	M	NP	Ra	IS,MG	F	Pisicivore / Invertebrate
45	Lesser Coucal *Centropus bengalensis*	R	TP	C	OC	D,E,G,H	Carnivore
46	Lineated Barbet *Megalaima lineata*	R	TP	C	GP,OC	A	Frugivore / Granivore
47	Long-tailed Parakeet *Psittacula longicauda*	R	OPB	C	GP,OC	A	Frugivore / Granivore
48	Olive-backed Sunbird *Cinnyris jugularis*	R	TP	C	GP,OC	B,D	Nectarivore
49	Oriental Magpie Robin *Copsychus saularis*	R	NP	C	GP,OC,LF,LMF	B,C,G,H	Insectivore / Invertebrate
50	Pied Fantail *Rhipidura javanica*	R	TP	C	MG,LF	A,B,D,F,G,H	Insectivore
51	Pied Triller *Lalage nigra*	R	TP	C	GP,OC	A	Insectivore
52	Plain-backed sparrow *Passer flaveolus*	R	NP	UC	GP,OC	G	Granivore / Insectivore
53	Purple-throated Sunbird *Leptocoma sperata*	R	TP	UC	GP,MG,LF	A,B,C,E,F,G	Isectivore / Nectarivore
54	Richard's Pipit *Anthus richardi*	R,M	NP	C	OC	A,B,E,F	Insectivore
55	Rufous Woodpecker *Micropternus brachyurus*	R	TP	C	LF	B,H	Insectivore / Invertebrate
56	Rufous-fronted Babbler *Stachyridopsis rufifrons*	R	TP	UC	LF,LMF	B	Insectivore / Frugivore
57	Rufous-tailed Tailorbird *Orthotomus sericeus*	R	TP	UC	MG,LF	A,B,G	Insectivore

Table 2 (*continued*)

58	Scaly-breasted Munia *Lonchura punctulata*	R	NP	C	GP,OC	F	Granivore
59	Scarlet-backed Flowerpecker *Dicaeum cruentatum*	R	TP	C	GP,OC,MG,LF	A,B	Nectarivore
60	Spotted Dove *Streptopelia chinensis*	R	NP	C	GP,OC	ALL	Frugivore / Granivore
61	Stripe-throated Bulbul *Pycnonotus finlaysoni*	R	TP	C	LF,LMF	B	Insectivore / Frugivore
62	Tiger Shrike *Lanius tigrinus*	M	TP	C	LF,LMF	C,F,G	Carnivores
63	Western Yellow Wagtail *Motacilla flava*	M	TP	C	OC	H	Insectivore/Invertebrate
64	White-bellied Munia *Lonchura leucogastra*	R	NP	UC	LF	C	Granivore
65	White-breasted Waterhen *Amaurornis phoenicurus*	R,M	GB	A	IS	G,H	Insectivore / Pisicivore
66	White-headed Munia *Lonchura maja*	R	NP	C	OC	E,G	Granivore
67	White-rumped Munia *Lonchura striata*	R	NP	UC	OC,LF,LMF	A,C	Granivore
68	White-throated Kingfisher *Halcyon smyrnensis*	R	TP	C	GP,OC	A,B,C,E,F,G,H	Pisicivore
69	Yellow-vented Bulbul *Pycnonotus goaivier*	R	NP	A	GP,OC	ALL	Frugivore / Insectivore
70	Zebra Dove *Geopelia striata*	R	NP	C	GP,OC	A,B,C,E,F,G,H	Granivore

Legend: Distribution status (I = introduced, R = resident, M = migrant); Protection Status (NP = not protected, TP = totally protected, GB = game birds, OPB = other protected birds); Degree of occurrence (A = abundant, C = common, UC = uncommon, RA = rare). Habitat association (GP = garden and parks; OC = open country; MG = mangrove; LF = lowland forest; LMF = lowland montane forest; UMF = upper montane forest; IS = inland swamp)

(17-19 June, 2008 and 12-15 May, 2009). Only babblers were recorded once (both on 10-13 March 2009 at station B).

The existence of the forest birds in Bachok indicate that they can survive in a degraded habitat with increased human activities, as long as the resources are available. A similar response was also shown by the forest birds in the tropical countryside of Costa Rica (Sekercioglu *et al*., 2007). Perhaps, prolonged destruction on the forested area surrounding the study area might have forced the forest birds to fully utilise any remaining habitat available for their survival. Consequently, they become less specific in choosing the habitat for survival and will eventually become more resilient to survive better in human dominated areas. Some of the forest birds that were recorded in this study, such as Chestnut-breasted Malkoha and Rufous Woodpecker, are commonly associated with degraded habitat like secondary forests in Johor (Peh *et al*., 2006) and Negeri Sembilan (Wong, 1986).

Degraded habitat generally supports fewer species than primary forest, especially in the short term; however, it is reasonable to expect that restoration of secondary habitat will allow some ameliorations of biodiversity loss. Tropical forest regeneration can be accelerated by planting fast-growing, fruit-producing trees, like figs, in the formerly forested areas. These trees attract birds and bats which will deposit seeds from the nearby forests onto the ground below. The dropping of these seeds will, in effect, return native forest species to the deforested patch.

Perhaps some degraded habitats (including those in Bachok) have high potential conservation values as in Costa Rican agricultural countryside (Sekercioglu *et al*., 2007) and are capable in providing adequate resources such as food, covers and nesting site for the survival of forest birds. If this is true, degraded habitat can significantly contribute towards the forest bird conservation programme where appropriate restoration measures are taken (Sekercioglu *et al*., 2007). However, without information on the nesting behaviour, feeding and foraging activity, as well as resource utilisation, a comprehensive conclusion about the value of the degraded habitat for forest birds cannot be made.

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Journal of Tropical Agricultural Science

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