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The Semen Characteristics of Pubertal West African Dwarf Bucks

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ABSTRACT

Semen was collected twice weekly between 0800 and 0900 hours from 6 healthy pubertal West African Dwarf (WAD) bucks for three months by electrical stimulation, and later evaluated and compared with values from healthy adult bucks of the same breed. Apart from semen colour, which was similar between pubertal and adult bucks, adult bucks were significantly superior ($p < 0.05$) to pubertal bucks in semen volume, mass activity, sperm progressive motility, sperm concentration, live sperm, total sperm/ejaculate and normal sperm morphology. In addition, pubertal buck semen also had significantly higher ($p < 0.05$) incidences of abnormal and dead spermatozoa. The incidence of coiled tails was however similar ($p > 0.05$) between the two age groups. The sperm concentration in the pubertal buck was highly significantly and positively correlated with total sperm/ejaculate ($r = 0.72$; $p < 0.001$), while semen volume was significantly but negatively related to the total sperm/ejaculate ($r = -0.37$; $p < 0.05$). Based on the findings of this study, it is concluded that pubertal buck semen, though inferior in quantity and quality to that of the adult, may be sparingly used for AI, and that good sires may be selected at puberty on the basis of the physical characteristics of their semen.

Keywords: Pubertal, bucks, semen characteristics, humid, tropical environment

INTRODUCTION

Goats are better adapted to hot environments than sheep and cattle (Coop, 1982; Valez-Nauer *et al.*, 1982), but are more susceptible to heat stress than sheep and cattle, as reflected by a rise in body temperature, outward signs of stress and a reduction in performance (Bianca & Kunz, 1978). Their importance in the livestock economy of people living in subsistence agriculture in the humid zone of West Africa has made them the species of choice for peasant farmers and for the supply of animal protein and other products in the sub-region. In Nigeria, the most popular genotype, i.e. the West African Dwarf (WAD) goat which supplies excellent quality meat, milk, skin and other products, has been adjudged as one of the most prolific in the world, with a remarkably high reproductive potential (Wilson, 1989; Gall *et al*., 1992). The WAD goat, however, is still largely unimproved. The improvement of the tropical breeds of goats generally and the WAD goat in particular for higher productivity as has been successfully done with goats in France (Leboeuf *et al*., 1998) will require information on the seminal characteristics of bucks. Earlier reports in the WAD buck concentrated on the adult bucks of the breed (Akusu *et al*., 1984; Ugwu

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& Orji, 1984; Bitto *et al*., 1988, 2000a, 2000b; Bitto *et al*., 2000c) and neglected pubertal bucks. However, the use of younger bucks for breeding purposes may save time and cost of management in raising bucks to adulthood. It is also a common occurrence in developing countries where goats are often mostly managed extensively to have pubertal bucks serve does successfully. Moreover, it has been reported that bucks exhibit sexual aggressiveness, penile development and permit intromission with good *libido* under tropical conditions at a very young age (Bongso *et al*., 1982). Thus, knowledge of the seminal characteristics of pubertal bucks is very useful in the early selection of sires for planned breeding programmes. In addition, older bucks constitute a problem as their *libido* and reaction time decline with increasing age (Butswat & Zaharaddeen, 1998). Inspite of our recent reports in pubertal bucks of the breed on sperm production rates and sperm storage capacity (Bitto & Egbunike, 2006a), testicular morphometry (Bitto & Egbunike, 2006b), some biochemical characteristics of spermatozoa and seminal plasma (Bitto *et al*., 2007) as well as the histometry of the testes (Bitto & Egbunike, 2008), information on the ejaculate characteristics of the pubertal West African Dwarf buck is still lacking. This study was therefore designed to characterize the seminal characteristics of the pubertal WAD buck in its native humid tropical environment, preparatory to the improvement of this particular breed.

MATERIALS AND METHODS

Animals and Management

Six healthy pubertal WAD bucks between 148-156 days in age and weighing between 9.10 and 12.00kg were used in this study. The pubertal bucks were obtained as kids bucks born to does at the Physiology unit of the Teaching and Research Farm of the University of Ibadan, where the experiment was conducted. They were weaned between 35 and 40 days of age and housed together in a group in a standard goat pen with concrete floor. The animals were fed a maize based concentrate ration supplemented with forage *ad libitum* and had access to cool clean drinking water at all time. They were allowed out for exercise early in the mornings on days with favourable weather.

Puberty

The preputial smear technique (Vandenberg, 1971; Egbunike, 1979) was used to determine the onset of puberty. Smears were taken every other day from each animal by gently and carefully rotating moistened cotton in the preputial pouch between the orifice and the glans penis. In each case, the adherent material was smeared on a clean dry glass slide and screened under a microscope for spermatozoa. This was continued until three consecutive positive readings from an animal were obtained. The cotton buds used were of the Bel de luxe-Hartman Ltd. brand.

Adult bucks which were meant for comparison had been previously used for breeding with satisfactory results. They were likewise housed in a standard goat pen in the same unit with adequate protection from prevalent ambient temperatures and other climatic conditions, apart from being maintained on a good plane of nutrition which had earlier been certified free from clinical abnormalities.

Semen Collection

As soon as the bucks had attained puberty, they were ejaculated twice weekly (on Tuesdays and Saturdays) between 0800 and 0900 hours, by the electro ejaculation technique for three months (January to March) as reported earlier by Bitto *et al*. (1988), Bitto *et al*. (2000a, 2000b), and Bitto *et al*. (2007). An electro-ejaculator probe was specially constructed to fit the relatively smaller rectum of the pubertal bucks. Semen was collected from 2 healthy adult WAD bucks, 22 and 28 months old with body weight ranging from 18.40 and 21.10kg for comparison (Bitto *et al*., 2007).

Semen Evaluation

Freshly collected semen samples were evaluated for colour, volume, mass activity, sperm progressive motility, sperm concentration, live/dead sperm, total sperm per ejaculate and abnormal sperm morphology by standard laboratory methods (Bitto *et al*., 2000a).

Statistical Analysis

Data obtained were subjected to the student's *t*-test (Steel & Torrie, 1980) for a comparison between pubertal and adult bucks. The linear relationships between the seminal characteristics in both pubertal and adult bucks were also assessed.

RESULTS

The physical characteristics of the semen of the pubertal buck, in comparison to those of the adult, are summarized in Table 1. Table 2 shows a summary of the comparison of sperm abnormalities between the age groups. Even though colour of the semen was consistently similar between the pubertal and adult bucks, the latter bucks were significantly superior ($p < 0.001$) to pubertal bucks in terms of their semen volume, mass activity, sperm progressive motility, sperm concentration, total sperm/ejaculate and normal sperm morphology. There was also a significant difference ($p < 0.01$) between the groups in the proportion of live sperm. Pubertal buck semen also had a significantly higher ($p < 0.01$) proportion of dead sperm than adult buck semen. In relation to abnormal sperm morphology, the semen of pubertal bucks had significantly higher proportions of detached normal heads ($p < 0.01$), bent tails ($p < 0.05$), double tails ($p < 0.001$), proximal cytoplasmic droplets ($p < 0.001$), distal cytoplasmic droplets ($p < 0.001$) and total abnormal sperm morphology ($p < 0.001$). There was however a similarity between pubertal and adult bucks in the incidence of coiled tails ($p > 0.05$).

The relationships between seminal characteristics in pubertal and adult bucks are presented in Tables 3 and 4, respectively. The sperm concentration in the pubertal bucks was highly, significantly and positively correlated with the total sperm per ejaculate ($r = 0.72$; $p < 0.001$). In the adult buck, however, the concentration of sperms was significantly and positively correlated to semen volume ($r = 0.66$; $p < 0.01$) and mass activity ($r = 0.53$; $p < 0.05$); highly, significantly and positively correlated to sperm motility ($r = 0.80$; $p < 0.001$) and total sperm/ejaculate ($r = 0.97$; $p < 0.001$). However, the concentration of sperm in the adult buck was found to be highly and significantly but negatively related to live sperm ($r = - 0.94$; $p < 0.001$) and normal morphology ($r = -0.72$; $p < 0.001$). Meanwhile, semen volume was highly, significantly and positively related to sperm motility ($r = 0.95$; $p < 0.001$) and total sperm/ejaculate ($r = 0.80$, $p < 0,001$). Mass activity was negatively and significantly correlated to live sperm ($r = -0.80$; $p < 0.001$). Sperm motility was highly positively significantly related to total sperm/ejaculate ($r = 0.88$; $p < 0.001$) and live sperm ($r = 0.87$; $p < 0.001$), but significantly and negatively related to dead sperm ($r = -0.43$; $p < 0.05$). Total sperm per ejaculation was also highly but negatively related to live sperm ($r = -0.92$; $p < 0.001$) and normal morphology ($r = -0.61$; $p < 0.01$). Moreover, live sperm was significantly related to normal morphology ($r = 0.50$; $p < 0.05$), and dead sperm was similarly highly significantly positively related to normal morphology ($r = 0.72$; $p < 0.001$).

DISCUSSION

The colour of pubertal bucks' semen obtained in this study, being similar to the adult buck, is consistent with the earlier observation and report in the adult WAD buck (Bitto *et al*., 1988); therefore, this is noted to be the characteristic of this particular genotype in its native environment.

The volume of pubertal bucks' semen in the present study was also lower than the values reported for the pubertal Damascus male goat (Elwishy & Elsawaf, 1971), but comparable with that reported at the onset of puberty in

Boer goats (Corteel, 1977). Pubertal buck semen volume obtained in the present study was also much lower than the values (much earlier) reported for the three breeds of pubertal rams (Skinner & Rowson, 1968). Semen volume, sperm motility and sperm concentration values obtained in both the pubertal and adult bucks in the present study were respectively lower than the corresponding values reported by Noran *et al*. (1998) for adult Katjang and Katjang x German Fawn goats. These differences are obviously due to genotype (breed) and species (in the case of sheep) differences in seminal characteristics. Semen volume, sperm concentration, total sperm/ejaculation and sperm motility obtained in the present study were lower in comparison to 13 month old Murciano-Granadina male goats in all four seasons of the year (Roca *et al*., 1992). These differences might be due to breed, age and environmental factors.

Sperm concentration in the pubertal WAD bucks in this study was much higher than the values reported for pubertal Damascus (Elwishy & Elsawaf, 1971) and Boer goats (Corteel, 1981). Meanwhile, the values of live sperm in both the pubertal and adult bucks were also higher than the corresponding values for adult Katjang and Katjang x German Fawn goats (Noran *et al*., 1998). Live sperm in the pubertal WAD bucks was also higher than that of Suffolk

TABLE 1
The semen characteristics of pubertal and Adult WAD bucks (means ± sem)

	Parameter	Pubertal buck (n=6)	Adult buck (n=2)	Level of significance
a.	Colour	Milky white	Milky white	$p < 0.001$
b.	Volume (ml)	0.20 ± 0.02	0.47 ± 0.02	$p < 0.001$
c.	Mass activity (1-5)	3.60 ± 0.08	4.25 ± 0.05	$p < 0.001$
d.	Progressive motility (%)	60.37 ± 1.00	74.66 ± 0.85	$p < 0.001$
e.	Sperm concentration ($x10^9$/ml)	0.53 ± 0.04	1.65 ± 0.07	$p < 0.001$
f.	Live sperm (%)	88.91 ± 0.61	91.88 ± 0.60	$p < 0.01$
g.	Dead sperm (%)	11.09 ± 0.61	8.12 ± 0.60	$p < 0.01$
h.	Total sperm /ejaculation ($x10^9$)	0.12 ± 0.01	0.79 ± 0.06	$p < 0.001$
i.	Normal morphology (%)	85.90 ± 0.72	94.15 ± 0.60	$p < 0.001$

sem = standard error of mean; ns = not significant ($p > 0.05$)

TABLE 2
The sperm abnormalities of pubertal and Adult WAD bucks (means ± sem)

	Parameter	Pubertal buck (n=6)	Adult buck (n=2)	Level of significance
a.	Detached normal heads (%)	3.01 ± 0.29	1.78 ± 0.26	$p < 0.01$
b.	Ben tails (%)	2.58 ± 0.30	1.36 ± 0.30	$p < 0.05$
c.	Coiled tails (%)	1.45 ± 0.23	1.66 ± 0.28	ns
d.	Double tails (%)	0.50 ± 0.00	0.00 ± 0.00	$p < 0.001$
e.	Proximal Cytoplasmic droplet (%)	3.58 ± 0.41	0.50 ± 0.00	$p < 0.001$
f.	Distal Cytoplasmic droplet (%)	2.98 ± 0.42	0.55 ± 0.04	$p< 0.001$
g.	Total abnormalities (%)	14.10 ± 0.72	5.85 ± 0.60	$p < 0.001$

sem = standard error of mean; ns = not significant ($p > 0.05$)

TABLE 3
The relationships between semen characteristics in the pubertal WAD buck

		8	7	6	5	4	3	2	1
1.	Sperm concentration	-0.27	0.72**	-0.19	-0.19	-0.21	0.01	-	-
2.	Volume	-0.37	0.20	-0.16	-0.16	0.09	0.23	0.23	
3.	Mass Activity	-0.16	0.11	0.33	0.33	0.06	-		
4.	Motility	0.06	0.06	-0.02	-0.02	-			
5.	Live (%)	0.18	-0.12	-1.00	-				
6.	Dead (%)	0.18	-0.12	-					
7.	Total sperm/Ejaculation	0.10				-			
8.	Normal Morphology	-							

*= p< 0.05; **= p < 0.01

TABLE 4
The relationships between semen characteristics in the pubertal WAD buck

		8	7	6	5	4	3	2	1
1.	Sperm concentration	-0.72***	0.09	-0.94***	0.94***	0.80***	0.53*	0.66**	-
2.	Volume	-0.04	0.40	-0.70**	0.80***	0.95***	-0.26	-	
3.	Mass Activity	0.80***	-0.42	-0.47*	0.33	0.01	-		
4.	Motility	-0.16	-0.43*	0.87***	-0.88***	-			
5.	Total Sperm/Ejaculate	-0.61**	-0.02	-0.92***					
6.	Live Sperm	0.50*	-0.22	-					
7.	Dead Sperm	0.72***	-						
8.	Normal Morphology	-							

*= p< 0.05, **= p < 0.01, ***= p < 0.001

rams in a similar age group (Skinner & Rowson, 1968). The differences may be due to factors like breed, method of semen collection, frequency of collection and probably the environment (Evans & Maxwell, 1987).

Likewise, the total sperm/ejaculation of the pubertal WAD buck in this study was found to be comparable to the lower limit but much higher than the upper limit ($0.123x10^9$/ml and $0.98x10^9$/ml respectively) reported for the pubertal Damascus goats (Elwishy & Elsawaf, 1971). However, the total abnormal sperm in the present study was highly comparable with that of pubertal Damascus goats (Elwishy & Elsawaf, 1971).

The differences between the pubertal WAD buck and other breeds of goats and rams in the seminal characteristics could be an expression of species, breed, nutritional status and environmental differences, as well as the differences in the methods used for semen collection and the frequency of the use of the animals. The highly significant differences between the pubertal and adult bucks in both semen quantity and quality are probably due to the differences in the development of the reproductive organs. These might have led to the obvious differences in the weights, sizes and capacities of the respective regions of the reproductive tract. Therefore, the two age groups would be expected to differ in some physiological processes, like sperm production, sperm maturation and sperm storage capacity, as well as the relative secretions of the various

accessory sex organs/glands which are all age dependent. However, it is worthy to observe that the total abnormal sperm morphology in the pubertal WAD buck obtained in this study (as in the adult buck) is within the acceptable range of not more than 20% of the semen acceptable for use in Artificial Insemination (AI).

The linear relationships between seminal characteristics, as well as the highly significant positive relationship between sperm concentration and the total sperm per ejaculation in the pubertal buck, are a normal trend. These indicate normal spermatogenic activities as well as other gonadal and extragonadal functions. The significant and positive relationships between sperm concentration, total sperm per ejaculate, sperm motility, semen volume and density in the adult buck are indications of the superiority of the adult bucks over the pubertal buck in terms of spermatogenesis, and other testicular and accessory organ functions. Meanwhile, the negative relationships between sperm concentration and live sperm and normal morphology probably express the differences between the quantitative and qualitative evaluations of the freshly collected semen, with sperm concentration being the actual number of spermatozoa per milliliter (ml), including dead and abnormal spermatozoa. In addition, the relationships between semen volume and motility, as well as the total sperm per ejaculation suggest high subjective fertility estimates, based on the semen volume in both the natural service and probably AI when semen is extended. The significant negative relationships between mass activity and live sperm, as well as the normal morphology in the pubertal buck, are explainable from the understanding of this particular criterion of semen evaluation. This being rather subjective, the wave motion of a semen sample is therefore dependent on a number of factors, but not limited to live and morphologically normal spermatozoa. The highly significant positive relationships between sperm motility and total sperm per ejaculation, live sperm and the significant negative relationship with dead sperm and the relationships of total sperm per ejaculate to live sperm are consistent with good quality semen in the adult buck. This status is where the pubertal buck would be expected to attain with age and good management.

CONCLUSIONS AND APPLICATIONS

In conclusion, the semen from the pubertal WAD bucks may be used sparingly in both natural mating and AI programmes and on the basis of their semen characteristics, good sires may be selected at puberty.

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Is a Mussel Processing Site a Point Source of Zn Contamination? Evidence of Zn Remobilization from Boiled Mussel, *Perna viridis*

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ABSTRACT

Sediment sampling in the Straits of Johore revealed that the surface sediments collected at a jetty near a mussel processing factory in Kg. Sg. Melayu had elevated Zn concentration in its first geochemical fraction; namely, easily, freely, leachable or exchangeable (EFLE) and its total concentration. This total Zn level in the sediment was comparable to the polluted sites on the west coast of Peninsular Malaysia. It was assumed that the tap water, in which mussels had been boiled, might have contained high levels of Zn which would then be released to the drainage system and finally emptied into the coastal waters where the jetty is located. In order to confirm this point source of Zn contamination, a laboratory study was designed to ascertain if the boiled mussels contained higher concentrations of metals compared to a control group. The laboratory results showed that distilled water, in which fresh mussel tissues had been boiled for 15 minutes, possessed significantly ($P < 0.05$) higher levels of dissolved Zn. In addition, Zn concentrations in the total boiled soft tissues and boiled shells of fresh mussel *Perna viridis* were significantly ($P < 0.05$) lower than the Zn levels before boiling, and this finding evidently showed that Zn in the mussel tissues was remobilized and thus released to the water. Therefore, these results supported the conclusion that the mussel processing factory at Kg. Sg. Melayu, which used tap water to boil the mussels before shucking, was a point source of Zn contamination in this area in the Straits of Johore.

Keywords: *Perna viridis*, Zn contamination, boiled mussels

INTRODUCTION

Before visiting a mussel (*Perna viridis*) processing factory, the authors did not have any idea that the factory, which is situated at a jetty in Kg. Sg. Melayu, could be point source of pollution for heavy metals into the nearby coastal environment in the Straits of Johore. An earlier study of the heavy metal concentrations in the sediment at the jetty at this site had revealed an elevated concentration of Zn (Yap *et al*., 2006a) which was comparable to the polluted sites on the west coast of Peninsular Malaysia (Yap *et al*., 2003a). This raised the question whether the tap water in which the mussels had been processed by boiling in a metal tank contained a high level of Zn (*Fig. 1A*). It was assumed that this mussel-boiled water would then be released into the drainage and finally emptied into the coastal waters where the jetty is located. The boiling of the fresh mussels is necessary to facilitate the shucking of the soft tissues out of the mussel shells before packaging (*Fig. 1B*). To confirm the hypothesis, the present study was designed to ascertain if the boiled mussels contained higher concentrations of metals. The objective of this study was to provide evidence for the above by

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comparing the concentrations of Cd, Cu and Zn between the boiled and unboiled soft tissues and the shells of *P. viridis*, including the water used to boil the mussels.

MATERIALS AND METHODS

Mussels and surface sediment samples were collected from the jetty of the mussel processing factory at Kg. Sg. Melayu which is located in the Straits of Johore. They were stored in a cool box upon transportation to laboratory in Universiti Putra Malaysia (UPM).

For the laboratory experiment, fresh total soft tissues were dissected from the shells of *P. viridis*. The mussels were separated by sex, according to the colour of gonadal tissues as described by Yap *et al*. (2006b). Total soft issues and shells were placed in a beaker of distilled water (500 mL) and heated in a water bath. The temperature was maintained between 95-97°C for 15 minutes. A control group was analyzed for metal concentrations without further processing. The dissolved water used to boil the mussel samples was collected before and after the 15

Fig. 1: Containers with tap water (A) used to boil the mussel Perna viridis *before they were shucked from their shells (B) for packaging. Photos were taken at the mussel processing factory at Kg. Sg. Melayu in the Straits of Johore*

TABLE 1
Comparisons of dissolved concentrations (mg/L) of Cd, Cu and Zn in the water used to boil the mussels between boiled and unboiled mussels using *t*-test in STATISTICA.

		Minimum	Maximum	Mean	Std error	Significance level
Female soft tissues	N= 5					
Cd	Boiled	0.001	0.024	0.014	0.004	P> 0.05
	Unboiled	0.005	0.025	0.020	0.004	
Cu	Boiled	0.001	0.016	0.006	0.003	P> 0.05
	Unboiled	0.001	0.025	0.006	0.005	
Zn	Boiled	0.014	0.160	0.093	0.025	P< 0.05
	Unboiled	0.020	0.036	0.027	0.003	
Female shells	N= 5					
Cd	Boiled	0.010	0.023	0.018	0.002	P> 0.05
	Unboiled	0.011	0.023	0.016	0.003	
Cu	Boiled	0.001	0.005	0.003	0.001	P> 0.05
	Unboiled	0.001	0.005	0.002	0.001	
Zn	Boiled	0.019	0.065	0.036	0.009	P> 0.05
	Unboiled	0.014	0.044	0.026	0.006	
Male soft tissues	N= 6					
Cd	Boiled	0.013	0.029	0.018	0.002	P> 0.05
	Unboiled	0.010	0.025	0.016	0.002	
Cu	Boiled	0.001	0.023	0.006	0.004	P> 0.05
	Unboiled	0.001	0.002	0.001	0.000	
Zn	Boiled	0.015	0.181	0.083	0.024	P< 0.05
	Unboiled	0.022	0.029	0.026	0.001	
Male shells	N= 6.					
Cd	Boiled	0.015	0.027	0.019	0.002	P> 0.05
	Unboiled	0.014	0.028	0.020	0.002	
Cu	Boiled	0.001	0.010	0.003	0.002	P> 0.05
	Unboiled	0.000	0.001	0.001	0.000	
Zn	Boiled	0.017	0.073	0.045	0.009	P< 0.05
	Unboiled	0.011	0.032	0.024	0.003	

Note: Values in bold indicate significant ($P < 0.05$) differences

minutes' boiling (i.e. as approximate time to boil the mussels at the factory). This treated water was further analyzed. Unboiled distilled water was used as a control. All the experimental treatments were conducted in triplicates. For this laboratory experiment, distilled water obtained from our laboratory was used instead of tap water. This was because distilled water possessed no metal contamination and this could be a scientific basis of valid interpretation, should there be any differences in the metal levels between the unboiled and boiled distilled waters.

All of the tissues of the mussels and sediment samples were dried at 105°C for 72 hrs until constant dry weight. Three replicates

TABLE 2
Comparison of the concentrations (µg/g dry weight) of Cd, Cu and Zn between the boiled and unboiled mussels in their total soft tissues and shells by gender using *t*-test in STATISTICA.

		Minimum	Maximum	Mean	Std error	Significance level
Female shells	N= 12					
Cd	Boiled	3.40	4.83	4.14	0.10	P> 0.05
	Unboiled	0.37	5.39	4.14	0.37	
Cu	Boiled	3.45	6.66	5.63	0.33	P> 0.05
	Unboiled	4.60	8.38	5.88	0.31	
Zn	Boiled	6.14	9.25	7.57	0.26	P< 0.05
	Unboiled	7.03	10.74	8.62	0.38	
Female soft tissues	N= 12					
Cd	Boiled	1.52	2.72	2.15	0.12	P> 0.05
	Unboiled	1.63	2.72	2.21	0.09	
Cu	Boiled	3.18	11.92	8.52	0.90	P> 0.05
	Unboiled	4.14	14.11	8.34	0.97	
Zn	Boiled	88.33	177.73	139.17	7.65	P< 0.01
	Unboiled	120.52	215.22	168.33	7.24	
Male shells	N= 15					
Cd	Boiled	2.74	4.76	4.06	0.13	P> 0.05
	Unboiled	0.39	5.30	4.24	0.30	
Cu	Boiled	3.50	6.66	5.53	0.24	P> 0.05
	Unboiled	4.14	6.34	4.97	0.17	
Zn	Boiled	5.49	9.81	8.18	0.34	P< 0.05
	Unboiled	7.48	14.23	9.68	0.47	
Male soft tissues	N= 15					
Cd	Boiled	1.40	2.89	2.14	0.12	P> 0.05
	Unboiled	1.25	2.59	1.96	0.11	
Cu	Boiled	3.10	12.43	8.59	0.83	P> 0.05
	Unboiled	4.01	14.35	8.24	0.81	
Zn	Boiled	98.68	170.02	134.60	6.61	P< 0.01
	Unboiled	132.18	220.12	166.67	8.15	

Note: Values in bold indicate significant (P < 0.05) differences

of each mussel treatment were then digested in concentrated nitric acid (AnalaR grade; BDH: 69%). The dried sediment samples were crushed using a mortar and pestle and sieved through a 63 µm aperture stainless steel sieve and shaken vigorously to produce homogeneity. For the analyses of the total Cd, Cu and Zn concentrations in the sediment samples, three replicates were analyzed by using direct aqua-regia method. About 1g of each dried sample was digested in a combination of concentrated HNO_3 (AnalaR grade; BDH 69%) and $HClO_4$ (AnalaR grade; BDH 60%) in the ratio of 4:1. The mussel and sediment samples were put into a hot-block digester first at low temperature (40°C) for 1 hour and they were then fully digested at 140°C for at least 3 hours. Later, the digested samples were diluted to a certain volume (40 mL) with double distilled water. After filtration, the prepared samples were determined for Cd, Cu and Zn, using an air-acetylene flame Atomic Absorption Spectrophotometer (AAS) Perkin-Elmer Model AAnalyst 800. The data were presented in µg/g dry weight basis.

For the analytical procedures of the four geochemical fractions of the surface sediments, sequential extraction technique (SET), described by Badri and Aston (1983) with a slight modification by Yap *et al.* (2002a), was used in this study. These four fractions were: 1), easily, freely, leacheable or exchangeable (EFLE), 2) acid-reducible, 3) oxidisable-organic, and 4) resistant.

To avoid possible contamination, all the glassware and equipment used were acid-washed, and the accuracy of the analysis was checked with the blanks and quality control samples made of standard solutions. The percentages of recoveries for the heavy metal analyses were 90-110%.

The dissolved concentrations of the metals in the water used to boil the mussels, between the boiled and unboiled water/mussel samples, were statistically analyzed using the *t*-test in STATISTICA (99' Edition).

TABLE 3
Comparisons of concentrations (µg/g dry weight) of Cd, Cu and Zn between the boiled and unboiled mussels in their total soft tissues and shells by combining male and female samples using *t*-test in STATISTICA; N= 27.

		Minimum	Maximum	Mean	Std error	Significance level
Shells						
Cd	Boiled	2.74	4.83	4.09	0.08	P> 0.05
	Unboiled	0.37	5.39	4.20	0.23	
Cu	Boiled	3.45	6.66	5.57	0.19	P> 0.05
	Unboiled	4.14	8.38	5.38	0.19	
Zn	Boiled	5.49	9.81	7.91	0.22	P< 0.05
	Unboiled	7.03	14.23	9.21	0.32	
Soft tissues						
Cd	Boiled	1.40	2.89	2.15	0.08	P> 0.05
	Unboiled	1.25	2.72	2.07	0.08	
Cu	Boiled	3.10	12.43	8.56	0.60	P> 0.05
	Unboiled	4.01	14.35	8.28	0.61	
Zn	Boiled	88.33	177.73	136.63	4.93	P< 0.01
	Unboiled	120.52	220.12	167.41	5.45	

Note: Values in bold indicate significant ($P < 0.05$) differences

RESULTS AND DISCUSSION

To compare between the boiled and unboiled control water samples in the female and male soft tissues of *P. viridis* shown in Table 1, the concentrations of Cd and Cu in both water types were found to be not significantly ($P > 0.05$) different. However, both the male and female soft tissues showed significantly ($P < 0.05$) higher levels of dissolved Zn in the boiled distilled water than in the unboiled water. The male shells also had significantly ($P < 0.05$) higher level of dissolved Zn in the boiled water than in the unboiled one. Similarly, the metal levels in the mussel samples from both the boiled and unboiled treatments were also analyzed to further understand this finding.

TABLE 4
Concentrations (mean ± standard error, µg/g dry weight) of Cu, Cd and Zn in the sediments collected from the jetty of the mussel processing factory at Kg. Sg. Melayu.

Sampling site	Mussel processing factory		
GPS reading:	N 01˚ 27.626' E 103˚ 42.335'		
Date of sampling:	11 August 2004		
Time of sampling:	9.26 am		
Weather:	Sunny, humid and hazy		
Location description:	1.Orang Asli housing area 2. The place where soft tissues are separated from the shells for packaging.		
Geochemical fractions	Cu	Cd	Zn
F1: EFLE	0.668 ± 0.011	0.186 ± 0.018	19.2 ± 0.215
F2: Acid-reducible	0.401 ± 0.011	0.228 ± 0.037	78.7 ± 0.715
F3: Oxidisable-organic	30.0 ± 1.38	0.864 ± 0.112	96.0 ± 0.203
F4: Resistant	35.7 ± 0.996	0.925 ± 0.342	88.3 ± 2.38
Summation (F1+F2+F3+4)= *SUM*	66.7	2.20	282.2
Non-resistant fractions (F1+F2+F3)= *NR*	31.1	1.28	193.9
$\frac{NR}{SUM} \times 100\%$	46.5%	58.0%	68.7%
Total metal concentration based on aqua-regia method	46.6 ± 2.38	2.83 ± 0.469	310 ± 14.9
Biological effects within ranges of metal concentrations in marine and estuarine sediments: Effects range low (ERL) (Long *et al.*, 1995)	34	1.2	150
Biological effects within ranges of metal concentrations in marine and estuarine sediments: Effects range median (ERM) (Long *et al.*, 1995)	270	9.6	410
Sediment Quality Values (SQV) for Hong Kong-Low (Chapman *et al.*, 1999)	65	1.5	200
Sediment Quality Values (SQV) for Hong Kong-High (Chapman *et al.*, 1999)	270	9.6	410

Note: F1= easily, freely, leachable or exchangeable (EFLE)

The boiled and unboiled control mussel samples [soft tissues and shells, Table 2] did not show any significant difference ($P > 0.05$) in the concentrations of Cd and Cu between both sexes. Interestingly for Zn, both the total soft tissues and shells of *P. viridis* [both male and female] had significantly ($P < 0.05$) lower levels of Zn after 15 minutes of boiling. To further strengthen the conclusion, when the male and female data are combined, similar results were found in which only the Zn levels in both the total soft tissues and shells were significantly ($P < 0.05$) decreased compared to the levels of Cd and Cu (Table 3). These results were inversely related to those observed in the water samples used to boil the mussels. This suggested that the losses of Zn in the soft tissues and the shells of *P. viridis* after 15 minutes boiling were due to the release into the water since significantly higher Zn level was detected in the treated water samples.

Heat treatment by either steam-blanching or baking of polluted fish leading to a decrease of the heavy metal content in all the tested fish parts was also reported by Atta *et al.* (1997). Similarly, Howarth and Sprague (1978) also reported that the cooking process decreased the protein content of the fish parts; hence, since heavy metals are bound to protein, this would be followed by the decreased concentrations of metals. Therefore, the decreased Zn concentration found in the soft tissues of *P. viridis* after 15 minutes of boiling is not surprising.

The observation of Zn release from the boiled mussel shells is interesting from a biomonitoring point of view. The results of the present study imply the possibility of Zn release from the mussel shells inhabiting natural environment if the mussels are submerged near surface water at a relatively higher water temperature for a long time. Therefore, although *P. viridis* shells have been suggested as a potential biomonitoring material for Zn (Yap *et al.*, 2003b, 2004), the seawater temperature could also influence the accuracy of the Zn interpretation. Thus, this abiotic factor should be taken into account when interpreting data. However, it can still be argued that the coastal waters would hardly reach up to 40°C and therefore the Zn bound to crystalline lattices of the shell structures could hardly be released (Yap *et al.,* 2003b).

The use of *P. viridis* as a biomonitor of heavy metals in the Malaysian coastal waters has been reported in some earlier studies (e.g. Yap *et al.,* 2003c, 2003d, 2004, 2006a). However, the phenomenon of Zn partial regulation that is thought to reduce the efficiency for the biomonitoring purpose is still very much debated. Therefore, the findings of the present study support the easy remobilization of Zn in the soft tissues of the *P. viridis* (Phillips, 1985; Yap *et al.,* 2002a, 2003c) which could possibly explain the partial regulation of Zn in this mussel species (Phillips, 1985).

Another interesting finding was the high concentration of Zn found in the surface sediment samples at the jetty of the mussel processing factory (Table 4). Based on the levels of Cu and Cd, the area could be considered as unpolluted compared to those reported by Yap *et al.* (2002b, 2003a), but the concentrations of Zn (310 μg/g dry weight) were comparable to those reported for the polluted sites, such as at Kuala Juru (306 μg/g dry weight) in Penang (Yap *et al.*, 2003a) and at a drainage (344.4 μg/g dry weight) in Serdang Metal Industrial site (Yap *et al.*, 2007). Besides the total Zn concentration, am elevated level was also evidenced in the first geochemical fraction of Zn; namely, easily, freely, leachable or exchangeable (EFLE) (Table 4).

In order to estimate the possible environmental consequences of Cd, Cu and Zn at the studied sites, the metals were compared to the Sediment Quality Guidelines of Effects Range Low (ERL) and Effects Range Median (ERM), as proposed by Long *et al.* (1995) (Table 4). The results showed that the concentrations of the three metals in Kg. Sg. Melayu were between the values for ERL and ERM. Similarly, compared to the Sediment Quality Values (SQV) of Cd, Cu and Zn for Hong Kong (Chapman *et al.*, 1999), the present Cd, Cu and Zn ranges fell between SQV-low and SQV-high for the three metals, and thus, indicating 'moderately polluted' status. Based on the percentage of non-resistant fraction

to the total summation of four geochemical fractions, however, the percentage for Zn is the highest (68.7%) among the three metals, indicating that most of the total Zn was related/ contributed by anthropogenic sources (Badri & Aston, 1983). Zn remobilization in *P. viridis* soft tissues, due to boiling process, had caused the release of Zn into the boiling water which was finally drained into the coastal water nearby.

An environmental concern could arise from the release of the mussel boiled water into the coastal waters. This could contaminate the surrounding waters, as well as increase the Zn bioavailability to the nearby coastal waters.

CONCLUSION

The presence of Cd, Cu and Zn was detected in both the shells and soft tissues of *P. viridis* which were sampled from a jetty situated at the Straits of Johore. Boiling the shells and soft tissues for 15 minutes hardly reduced the concentrations of Cd and Cu in both parts of the mussels. However, the concentrations of Zn significantly decreased in the shells and soft tissues after the boiling process. One plausible explanation is that the Zn was released into the boiled processing water and then discharged into the coastal waters. Hence, the findings of the present study showed that the mussel processing factory at Kg. Sg. Melayu was a point source of Zn contamination in the Straits of Johore. However, it should be noted that the Zn release could depend on the boiling conditions (time, temperature and medium of cooking) which should merit further studies.

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The Ranging Behaviour of *Tyto alba* in Oil Palm under Baiting with Anticoagulant Rodenticides, Warfarin and Brodifacoum and a Biorodenticide *Sarcocystis singaporensis* (Zaman & Colley, 1975)

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ABSTRACT

This study investigated the ranging behaviour of *Tyto alba* in oil palm under three different rodenticide applications. For this purpose, four treatment plots were established in the FELCRA oil palm plantation in Seberang Perak. Three plots were baited each with warfarin, brodifacoum and a protozoan based biorodenticide, *Sarcocystis singaporensis*, plus a fourth non-baited control plot. For each plot, a pair of *T. alba* was attached with radio transmitters and tracked for three nights with a radiotracking equipment. Radio locations were plotted on a 1:66 scale map. These radio locations were used to determine the home range size, the core area size, the mean distance moved from one radio location to the next and the furthest radio location from the nest box or the centre of activity. Data were analysed with the help of the software BIOTAS. The home ranges were analysed using the method of Minimum Convex Polygon (MCP), the Harmonic Mean (HM) and the Kernel estimator. The home range sizes of the chemical rodenticide areas were consistently larger than the biorodenticide and the non-treated control areas. For males, the home sizes calculated using the MCP method were 60.51 ha; 36.95 ha; 18.19 ha and 15.22 ha for the brodifacoum, warfarin, control and biorodenticide treated plots, respectively. As for the females, the corresponding home range sizes were 69.39 ha, 52.50 ha, 28.80 ha, and 49.85 ha. Meanwhile, the home range sizes of the females were significantly larger than those of the males when calculated using the MCP and HM methods. The core area size, which is conventionally treated as the defended area around the nest box, yielded male core area sizes of 16.43 ha, 9.0 ha, 4.48 ha and 1.39 ha for brodifacoum, warfarin, control and biorodenticide treated plots respectively, based on the MCP method. The corresponding core area sizes for the females were 28.55 ha, 37.17 ha, 11.21 ha and 19.02 ha. The females tend to move over a longer distance compared to that of the males; however, the mean distances travelled by the females and males were not significantly different. The data suggest that the furthest radio locations of the females from the nest box were greater than that of the males in all the treatment plots. The difference between the furthest radio locations of the females and males were significant. These data suggest that in areas treated with chemical rodenticide, *T. alba* has to engage in greater exploratory flight resulting in larger home range size, core area size and greater distance between the radio locations to secure enough prey to meet their energetic demands.

Keywords: *Tyto alba*, rat control, anticoagulant rodenticides, *Sarcocystis singaporensis*, radio-telemetry

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INTRODUCTION

Secondary poisoning to non-target animals can be primary, i.e. the exposure to poisoned baits, when non-target species consume them directly; secondary, when predators eat poisoned prey; or tertiary, when these predators were in turned consumed by a top predator (Smith *et al.*, 1990). There have been reports of secondary poisoning of predatory birds from the use of brodifacoum to control rats in many parts of the world. Eastern Screech-Owls, *Otus asio*, were found dead after exposure to brodifacoum, which had been applied in orchards (Merson *et al*., 1984; Colvin *et al.,* 1987). In New Zealand, the woodhens, *Gallirallus australis australis* and *Gallirallus australis scotti* were found dead after consuming rats that had fed on bait containing brodifacoum (Taylor, 1994). In Britain, the issue of secondary exposure and poisoning by second-generation rodenticides was highlighted in the 1980s as a part of the National Predatory Bird Monitoring Scheme via a long-term study in which residues were detected in barn owl *Tyto alba* carcasses which had been collected from throughout the country (Newton *et al.*, 1990). There have been reports of *T. alba* decimated in oil palm in Malaysia as a result of secondary poisoning from second generation rodenticide. Berny *et al*. (1997) suggested that poisoned rodents were more vulnerable and more easily captured than their unexposed counterparts because of their slower reactions, thereby increasing the chance of capture by predators such as, the barn owl. This will certainly enhance the exposure of anticoagulant rodenticides to raptors and carnivores.

The risk of secondary poisoning from chemical rodenticides prompted the search for an alternative approach that would not disrupt natural predation. In the recent years, the use of biorodenticides has found its practical application in the field for rodent control. One of the most effective biorodenticide to date is *Sarcocystis singaporensis,* a protozoan parasite. Zaman (1976) stated that *S. singaporensis* has been extensively studied for more than 25 years on its potential as a biorodenticide. It is a parasitic unicellular organism of the phylum Apicomplexa. The main hosts of the parasite include boid snake (*Phyton reticulatus)* and rodents of the genera *Rattus* and *Bandicota.* This particular parasite is not effective for or harmful to a wide range of birds, reptiles, mammals including primates, as well as other members of the rodent family, *Muridae*. Previous laboratory and field studies revealed that the infection of wild rats with high numbers of infective stages of *S. singaporensis* induced mortality of up to 100% in the laboratory and 70%-90% in the field after 10-14 days (Jakel *et al*., 1999). This parasite induces a fatal pneumonia in rodents once infection with sporocysts exceeds a certain threshold (Wood, 1985). Jakel *et al*. (2006) have shown that tactical infection of field rats with *S. singaporensis* is highly effective and economically competitive to poison baits in ricefields.

Since *S. singaporensis* has a specific host, it can be a viable option for a sustainable biological control of rodents using barn owl in oil palm. *T. alba* a vagrant species at the turn of the century is now a common resident in oil palm. Basri *et al*. (1996) reported that *T. alba* were widely established in oil palms and their population reaching some 15% of areas in Peninsular Malaysia by the early 1990s. The owls feed almost exclusively on rats, for instance, it makes up 99.4% of their diet in one study (Smal, 1990), it had gone from ‘‘rare’’ to ‘‘common’’ in Malaysia (Duckett & Karuppiah, 1990).

Warfarin has been the main rodenticide applied in most oil palm plantations in Malaysia, in combination with the natural propagation of *T. alba.* However, with reports of rats showing resistance towards warfarin, the second generation anticoagulant, particularly brodifacoum, is increasingly employed. While there has been no apparent evidence of secondary poisoning from warfarin, the detrimental effects of brodifacoum on *T. alba* have been documented (Mendenhall & Pank, 1980). Apart from the known and unknown risks from secondary poisoning, the application of rodenticides may have other effects, including the behaviour of predators. In particular, they may influence

the foraging behaviour of the owls, which can have a far reaching consequence in the long run. In this study, radiotelemetry was employed to compare the foraging behaviour of owls under the application of baiting with warfarin, brodifacoum and biorodenticide. A number of parameters, including home range size, core area size, mean distance moved and furthest distance moved away from the nest, were used to compare the effects of rodenticides to *T. alba* behaviour of these rodenticides. This study has provided some insights into the impact of rodenticide and how it may influence the sustainability of the barn owl natural propogation programme for a viable rat control in oil palm.

MATERIALS AND METHODS

The study was conducted at the FELCRA oil palm plantation in Seberang Perak, Perak, from March to May 2009 and from July to September 2009, both during the breeding seasons when rat damage to oil palm was greater than 5% at the plantation.

Baiting with Rodenticides

Four treatment plots, with six nest boxes each, were established in this study. The area for each plot is not less than 100 ha. Each plot is separated between three and five km from one another. Three plots were baited with warfarin, brodifacoum and the biorodenticide, based on *Sarcocystis singaporensis*, respectively. The fourth was left untreated and served as the rodenticide-free control plot. Since the nest boxes were not evenly distributed, the average density of the nest boxes worked out to be one box for every 25 ha. The first baiting campaign with warfarin and brodifacoum was carried out on 20-25th October 2008 and the second baiting campaign was carried out on 10-12th March 2009. Baiting with biorodenticide was carried out on 25-27th January 2009. The baits were placed at the base of each palm tree. In the first campaign, a single round of baiting was carried out, while two baiting rounds were conducted in the second baiting campaign. The baits were placed at the base of each palm tree in each of the designated plots.

Radio Telemetry

In this study, radio telemetry was used to detect the locations of owls in real time and delineate individual home range size. The radio telemetry set was comprised of a radio receiver Model CE – 12 (Custom Electronics of Urbana Inc), with a frequency range coverage of 150 – 152 MHz. The transmitters used were surface-mounted design, equipped with a 3-3.5 V cells. Signals emitted by the transmitters were detected with the help of a 3-element Yagi antenna. The frequency employed for this study was in the range of 150-151 MHz. Meanwhile, the 3-element Yagi antenna is suitable for the frequency above 140 MHz and for tracking on foot. This antenna has the advantage, based on its signal reception pattern of distinguishing between true and reverse bearing (Kenward, 2001).

A pair of *Tyto alba* nesting in one of the nest boxes in each treatment plot was captured and tagged with a radio transmitter. This would allow a comparison between the mating pairs. The occupancy rate at the time of the radio telemetry exercise was in the range of 50 to 66% (three or four boxes occupied in each plot). This would reduce the effects of different densities of barn owl on home range size. The transmitters were harnessed and mounted as backpacks on each *T. alba* and were pre-set to emit signals at unique frequencies. By tuning the radio receiver to the desired frequency and following the path of the strongest signal, the position of the owl was ascertained on the ground. Each bird was followed for three nights, i.e. from 1900 hrs to 0700 the following day. The radio locations of the owls were recorded at an hourly interval. Radio tracking commenced four to five days after the second baiting campaign. It has been established in most wildlife radio tracking studies that at least 35 fixes are required since any additional radio locations add little to the home range area (Kenward, 1987).

Home Range Analysis of Barn Owl

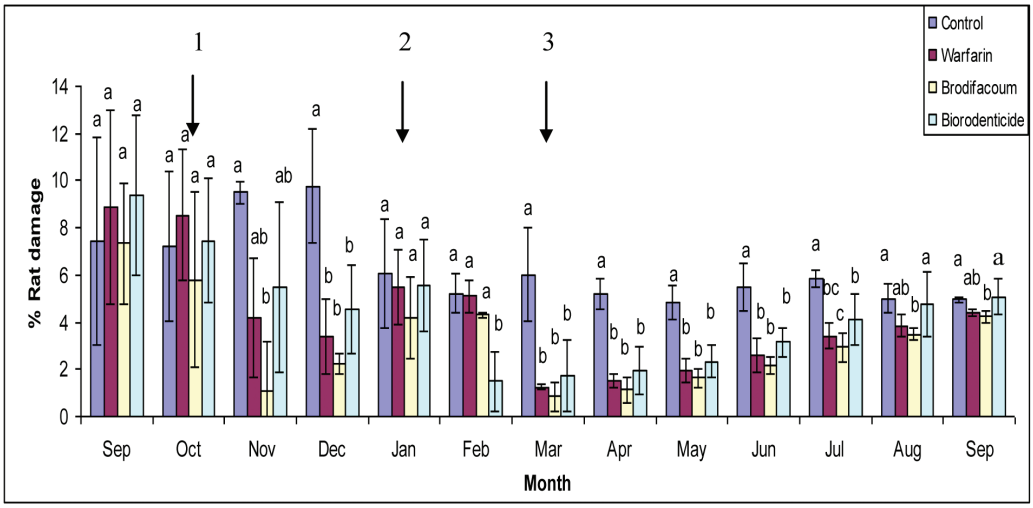
The home range and the core area size were calculated using the Minimum Convex Polygon (MCP) and the Harmonic Mean (HM) methods. The dataset was analysed with the help of BIOTAS, an ecological software solution. The MCP method, which simply calculates the area of the polygon formed around the outermost radio locations, has been the most commonly employed method to represent range size and shape (Harris *et al*., 1990). Meanwhile, the HM method allows the demarcation of the 'core area' which can be assumed as the defended perimeter, if territoriality is exhibited by the owls. By convention, 95% and 50% of the utilized areas calculated were regarded as the home range and the core area, respectively.

Harris *et al*. (1990) suggested using at least two home range estimators with any dataset; one of which should be the MCP because it would be used to compare with other home range studies. Meanwhile, Seaman *et al*. (1999) reported that out of the home range studies published from 1980 to 1997, 87% used the MCP method, the HM estimator (22%), the kernel method (7%), and the bivariate normal ellipse method (7%). In this study, the MCP and the HM methods were employed, along with a third which is the most recently developed kernel method, using the statistical technique that is increasingly used in home range studies. The results from the BIOTAS output would be multiplied with the actual distance on the ground so as to estimate the actual home range size of *T. alba.* The radio locations were first plotted on a 1cm x 1cm graph paper to determine their coordinates; the area 1cm x 1cm (1 cm^2) on the graph represents 66.67m x 66.67m (4445 m^2 = 2.25 ha) in the field.

RESULTS

Rat Damage

The percentage of rat damage is shown in *Fig. 1*. After the first baiting campaign in the biorodenticide and warfarin treated plot, rat



1. First baiting campaign – all rodenticides
2. Second baiting campaign – biorodenticide only
3. Second baiting campaign – warfarin and brodifacoum

Fig. 1: The mean Percentage of rat damage in all the treatments from September 2008 to September 2009. Arrows indicate rodenticide application. Different letters indicate significant difference

TABLE 1.1
Home range size* of males.

Treatment	Home range size (ha.)		
	MCP	HM	KERNEL
Control	18.19	27.43	33.46
Warfarin	36.95	59.14	78.19
Brodifacoum	60.51	115.09	147.37
Biorodenticides	15.22	46.63	25.50

*The home range size is based on the calculations from 95% of radio fixes (to reduce the effects of outliers). In the MCP method, the furthest fix was ignored.

TABLE 1.2
Home range size* of females

Treatment	Home range size (ha.)		
	MCP	HM	KERNEL
Control	28.80	41.84	58.22
Warfarin	52.50	91.64	143.12
Brodifacoum	69.39	160.52	167.33
Biorodenticides	49.85.	70.42	104.82

*The home range size is based on the calculations from 95% of radio fixes (to reduce the effects of outliers). In the MCP method, the furthest fix was ignored.

damage was found to decrease from 7.46% to 5.47%, and from 8.55% to 4.18%, respectively. These reductions were substantial compared to the control plot (9.49%) although they were not significantly different ($F = 4.65$; $p = 0.052$). In the brodifacoum plot, the level of damage decreased to 1.11% from 5.80%, which was significantly lower than the control plot, although not significantly different from the warfarin and biorodenticide treated plots ($F = 4.65$; $p = 0.052$). When two baiting rounds were carried out in the second campaign, rat damage was found to have substantially decreased from 5.56% to 1.48% in the biorodenticide treated plot, and this was significantly lower than the control plot (5.20%) ($F = 14.84$; $p < 0.01$). Meanwhile, the rat damage increased gradually after baiting, but it remained low (4.13%), and was still significantly lower compared to the control plot (5.86%) ($F = 15.46$; $p<0.01$) six months after baiting. Similarly, rat damage in the warfarin treated plot increased gradually after baiting. Compared to the biorodenticide plot, however, the level of damage in the warfarin plot one month after baiting was 4.41%, and this was not significantly different from the control plot (4.97%). In the brodifacoum plot, the damage was decreased to 0.84% from 4.32%; a reduction which was significantly lower than that of the control plot (6.02) ($F = 19.79$; $p < 0.01$).

Home Range Size

Signals from all the radio-tagged individuals were successfully detected by the radio receiver. Only 95% of the radio fixes were included, while the furthest point was ignored to minimize the influence of the outliers on the home range size. The estimated home range and core area size are summarized in Tables 1.1 and 1.2 for the males, and in Tables 2.1 and 2.2 for the females.

The home range sizes estimated by the three methods yielded the following results. For the males, the estimated home range size

TABLE 2.1
Core area size (50%) of males

Treatment	Core area (ha.)		
	MCP	HM	KERNEL
Control	4.48	5.91	2.99
Warfarin	9.0	15.65	5.97
Brodifacoum	16.43	22.06	36.25
Biorodenticides	1.39	3.27	3.32

Estimation of Core area is based on 50% of radio fixes (Selection of radio fixes by the software is based on different criterion for each estimator)

TABLE 2.2
Core area (50%) of females

Treatment	Core area (ha.)		
	MCP	HM	KERNEL
Control	11.21	7.46	12.91
Warfarin	37.17	20.74	35.54
Brodifacoum	28.55	24.44	31.72
Biorodenticides	19.02	24.06	26.73

Estimation of Core area is based on 50% of radio fixes (Selection of radio fixes by the software is based on different criterion for each estimator)

determined by the MCP method were 15.22 to 60.51 ha, followed by 27.43 to 115.09 ha for the HM method, and 25.50 to 147.37 ha for the fixed kernel range estimator. For the females, the home range size estimated from the three methods yielded the home range sizes of 28.80 to 69.36 ha, 41.84 to 160.42 ha, and 58.22 to 167.33 ha, respectively. The home range sizes, estimated using the MCP method, were smaller than the other two methods for both the sexes. The home ranges delineated by the MCP method are shown in *Fig. 2 – 5*.

The data from all the three methods consistently showed that the range size of the individuals in the areas treated with chemical rodenticides was larger than those of the untreated control and biorodenticides treated areas. Meanwhile, the home range sizes of the females were significantly larger than the males, as calculated using the MCP (*t*-test, P = 0.028) and the HM (*t*-test, P = 0.019) methods, but they were not significantly different when calculated by the kernel estimator (*t*-test, P = 0.071).

Core Area Size

The core area sizes of individual owls are shown in Tables 2.1 and 2.2. The percentages of the core area to the home range size are shown in Tables 3.1 and 3.2. For the males, the estimated core area, using 50% MCP ranging between 1.39 and 16.43 ha, corresponded to 9% and 27% of the home range size, respectively. The core area size determined by 50% HM ranged from 3.27 to 22.06 ha, which corresponded to 15% and 19% of the home range sizes, respectively. The kernel estimator yielded core area sizes of 2.99 to 36.25 ha, which corresponded to 8% and 24% of the home range sizes, respectively. The core areas for the males were consistently smaller in the untreated control and biorodenticide treated areas, and these corresponded to 4.48 and 1.39 ha, based on the 95% MCP method, followed by 5.91 and 3.27 ha (the 95% HM method), and 2.99 and 3.32 ha (the 95% kernel estimator). In comparison, the core area for the males in warfarin and brodifacoum treated areas were 9.0

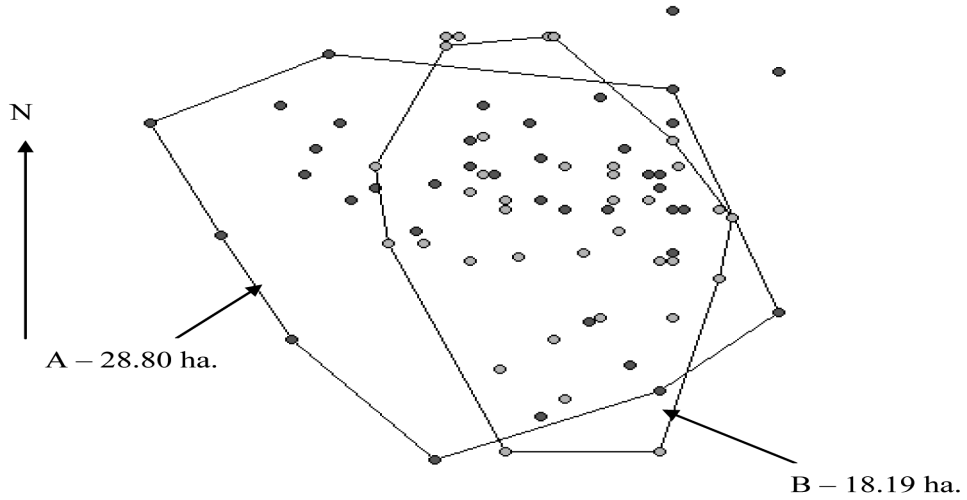


Fig. 2: Home Range of owls in the control plot using the MCP method: A – Female; B – Male (not drawn to scale)

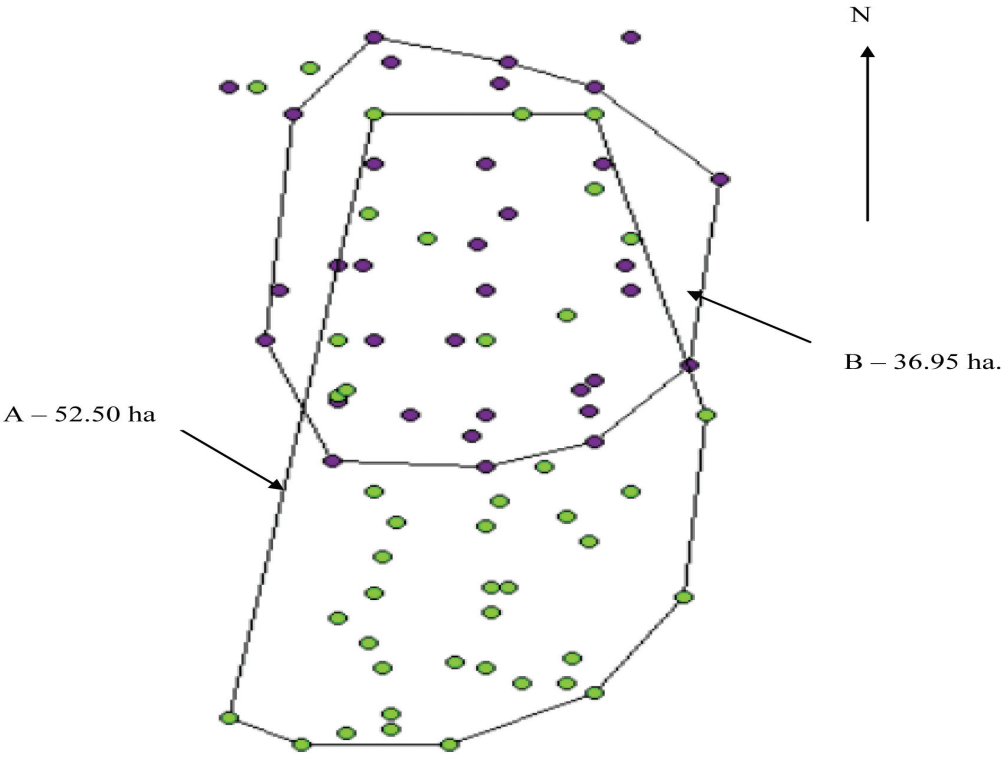


Fig. 3: Home Range of owls in warfarin Treatment plot using the MCP method: A – Female; B – Male (not drawn to scale)

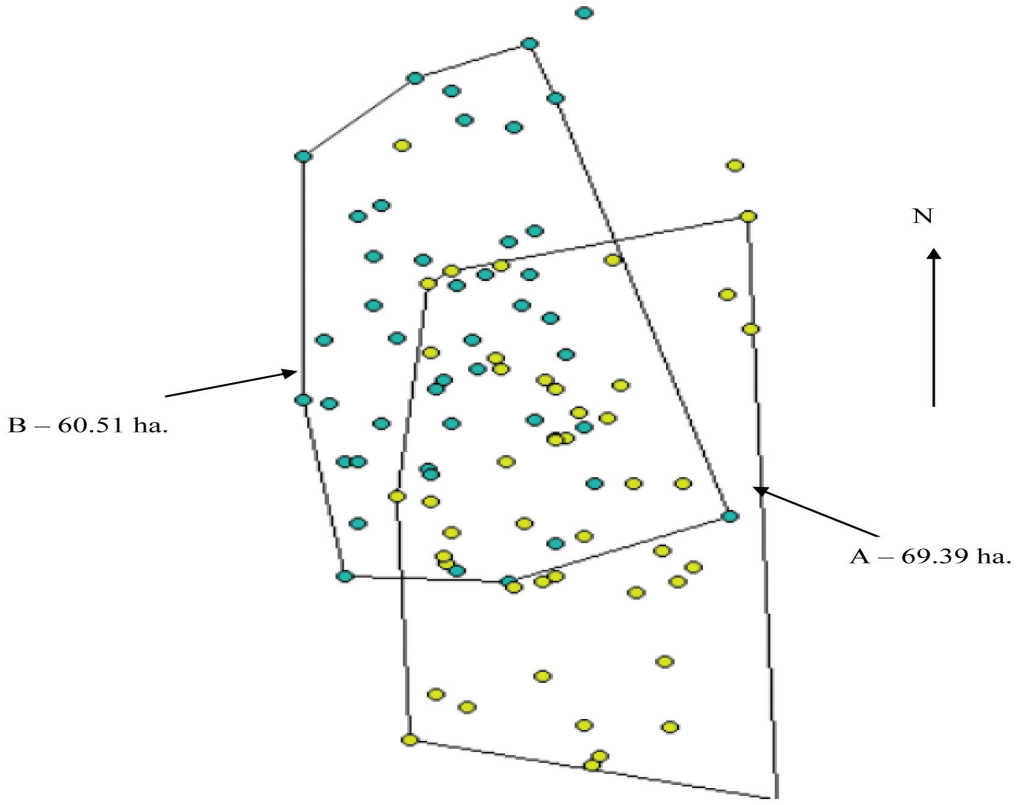


Fig. 4: Home range of owls in brodifacoum treatment plot using the MCP method: A – Female; B – Male (not drawn to scale)

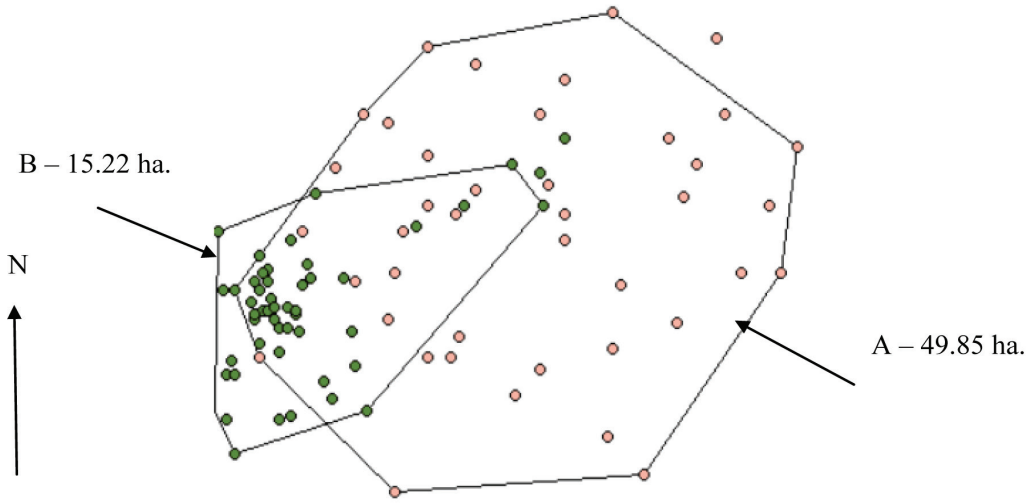


Fig. 5: Home range of owls in biorodenticide treatment plot using the MCP method: A – Female; B – Male (not drawn to scale)

TABLE 3.1
Percentage of the core area size of males

Treatment	Percentage of core area to home range size (%)		
	MCP	HM	KERNEL
Control	24.65	21.54	8.95
Warfarin	24.36	26.46	7.64
Brodifacoum	27.15	19.17	24.60
Biorodenticides	9.17	7.02	13.02

Table 3.2
Percentage of the core area size of the females

Treatment	Percentage of core area to home range size (%).		
	MCP	HM	KERNEL
Control	38.90	17.82	22.18
Warfarin	70.80	22.63	24.83
Brodifacoum	41.15	15.23	18.96
Biorodenticides	38.17	34.17	25.50

and 16.43 ha, based on the MCP method, 15.65 and 22.06 ha (the HM method), and 5.97 and 36.25 ha (the kernel estimator). The core areas for the females were consistently smaller in the untreated control plot by the MCP, the HM and the kernel estimator, with 11.21, 7.46 and 12.91 ha, respectively. However, the core area sizes of the females in the rodenticide treated areas were larger.

As for the females, the core area estimated by the 50% MCP method ranged between 11.21 and 28.55 ha, which corresponded to 38% and 41% of the home range sizes determined using the 95% MCP method. The core area calculated using the 50% HM ranged between 7.46 and 24.44 ha and this corresponded to 18% and 15% of the home range sizes calculated with the 95% HM method. The core area, calculated by the kernel estimator, ranged between 12.91 and 35.54 ha, and this corresponded to 22% and 25% of the home range size estimated with 95% kernel estimator.

A comparison between the males and females showed that the core area of the latter group were significantly larger than their male counterparts, when estimated with 50% MCP (*t*-test, P = 0.04), but not significantly different from HM (*t*-test, P = 0.06) and the kernel estimator (*t*-test, P = 0.10). Meanwhile, a comparison of the percentages of the core area to home range between the males and females showed that they were not significantly different, either using the MCP method (*t*-test, P = 0.12); the HM (*t*-test, P = 0.25) or the kernel estimator (*t*-test, P = 0.08).

Mean Distance Moved between Radio Locations and Furthest Radio Locations from Nestbox

The mean distance moved between the radio locations for the males and females are shown in Table 4.1. The females had the tendency to move over a longer distance compared to males. The mean distance moved by the females and males ranged from 281.18 m to 453.03 m, and from 184.09 m to 449.67 m, respectively. Thus, the mean distance travelled by the females and males were not significantly different (*t*-test = 0.072, P > 0.05). This could be attributed to the small sample size, being a female and a male each for

TABLE 4.1
The mean distance travelled between radio locations

Treatment	Male (m)	Female (m)
Control	228.96	281.18
Warfarin	327.62	400.33
Brodifacoum	449.67	453.03
Biorodenticides	184.09	384.39

TABLE 4.2
The furthest radio locations from the nestbox

Treatment	Male (m)	Female (m)
Control	515.36	578.67
Warfarin	700.03	920.71
Brodifacoum	1076.05	1474.74
Biorodenticides	673.37	774.71

each treatment plot. Table 4.2 summarizes the furthest radio locations from the nest box, i.e. a measure of how far a bird flew away from the nestbox. The furthest distance moved from the nest box for each treatment for the females were 578.67m for the control untreated plot, 920.71 m for the warfarin treatment plot, 1474.74 m for the brodifacoum treatment plot and 774.71m for the biorodenticide treatment plot. The data suggest that the furthest radio locations of the females from the nest box were greater than that of the males in all the plots. For comparison purposes, the furthest radio locations from the next box for the males were 515.36m, 700.03m, 1076.05 m and 673.37 m, respectively. The difference between the furthest radio locations of the females and males was found to be significant (t-test = 0.0402, $P < 0.05$).

DISCUSSION

The home range size of individual owls radio tagged in the chemical rodenticide areas was consistently larger than the biorodenticide and the non-treated control area. This is evidently obvious as rodents in the warfarin and brodifacoum treated areas could be suggestively lower in density (as reflected by the damage levels in *Fig. 1*) compared to the other treatment plots, indicating that the low rat population is associated with larger home range size of barn owl. Village (1982) showed that home range of kestrel was inversely correlated with vole abundance, i.e. low vole population size was associated with large home range size and vice versa. Depletion of rat population from direct feeding of baits could be the plausible reason why the home range sizes of owls were substantially larger. It reflects the greater exploratory flight the owls have to engage before encountering a potential prey. Meanwhile, the home range size of owls radio-tracked in biorodenticide treated area was smaller and in fact almost comparable to the control untreated plots. This could be explained by the delayed action of the onset of death. In contrast, Wood *et al.* (1989) found that the LFP_{50} for *R. tiomanicus* fed with warfarin was 3.08 days (1.38 – 9.08) for the females and 1.22 days (0.38 – 4.16) for the males. For single feeding anticoagulant like brodifacoum, death would be much quicker. Although rats exposed to biorodenticide baits did not succumb as fast as rats that had consumed single feeding chemical rodenticides, particularly the second generation

anticoagulant, they were not as active as when the parasites started to induce pneumonia, i.e. 10 – 14 days post consumption (Jakel *et al.,* 1999). This is because the parasite multiplies in the endothelial cells of blood vessels of the rat, thereby perforating the vessels once it leaves the host cells (Jakel *et al.,* 1996). Therefore, they become easy preys for the owls to spot on and this explains the substantially smaller home range, which is quite similar to the baseline home range size in normal hunting condition, i.e. the non-treated areas.

The home range sizes of females were consistently larger than those of the males in all the treatment plots as estimated by all the three methods. This could be explained by the females being more active at the onset of the breeding season. A study by Taylor (1994) showed that the females had to accumulate substantial body fat reserve to prepare for the nesting and incubation period. Sufficient fat reserve is crucial to produce a viable clutch size and to improve reproductive output. Taylor (1994) found that clutch size was related to the body weight of the female immediately before laying. The larger home range size reflects the higher rate of predation by a female compared to a male which only hunts for individual metabolic requirements.

Core area is a reflection of the area around the centre of activity within the general home range area. It could be defended or otherwise depending on the food resources. In barn owls, the core areas are typically associated with the nest box. If territorial behaviour is maintained than the core area is mutually exclusive and actively defended from encroachment by other individuals. According to Jason *et al.* (2005), barn owls usually do not actively defend their foraging territories from other owls, but they will defend the immediate area around their nests. In this study, this particular aspect could not be ascertained as a number of owls from neighbouring boxes will have to be radio-tracked simultaneously. This is an indication that the males in the rodenticide-treated areas have to maintain a larger core area size to secure sufficient prey.

The mean distance between the radio locations was also quite consistent with the home range and the core area data. The mean distances travelled between the radio locations for the females were further than those of the males for all the treatments, illustrating a higher hunting activity of the females. The same argument applies i.e. the females have to accumulate sufficient body fat for egg production and subsequent incubation. Higher energetic requirements demand higher food intake. Assuming that the males and females hunt with equal efficiency, the latter have to move over a longer distance per unit time to secure more prey than the males. The furthest radio locations from the nest box for the females were also greater than those of the males. This supports the argument that the females would venture further than the males from the centre of activity, i.e. the nest box in search of greater quantities of prey. According Newton (1979), females must also build up enough reserves for use during incubation to act as an insurance against temporary food shortage, while Perrins (1970) suggested that females started breeding as soon as they had accumulated enough reserves of energy and nutrients to produce eggs. Thus, insufficient prey will influence female barn owl's ability to accumulate adequate body reserves which consequently lead to failure to start breeding (Taylor, 1994).

The comparison between the treatment plots is also consistent with the home range and the core area data. The mean distance travelled between the radio locations were the highest in the warfarin- and the brodifacoum-treated areas for both the males and females. Meanwhile, the densities of the rats in these plots, where chemical rodenticides had been applied, were lower than the control untreated plot. Chemical rodenticides, especially brodifacoum has been proven to be effective against rat (Wood *et al.,* 1989) as it only needs a short time to inflict death to rats when baits have been ingested. In the warfarin- and brodifacoum-treated areas, rat damage was successfully suppressed to below 2% and 1% respectively from around 5% before baiting was done. The lower rat population

would result in a larger home range size of barn owl where they had to travel over a larger area to look for prey. The data for biorodenticide treated area seemed to match those of the control non-treated area. This is probably due to the rats taking longer time to succumb from the onset of pneumonia. As they grew weaker, the owls had a better chance of hunting them down. Comparing the furthest radio locations from the nest box shows that of the distance in the brodifacoum treated area was twice that of the biorodenticide area for both the males and females. Rats exposed to brodifacoum succumbed much earlier than those who were exposed to biorodenticide. Therefore, the higher and faster rate of mortality in the brodifacoum treated area resulted in a substantially lower rat density compared to the biorodenticide treated area. Consequently, the owls had to cover a much greater distance from the nest box to meet their food requirements.

Jason *et al.* (2005) estimated the individual home ranges of barn owls to be as large as 3174 ha and as small as 151 ha depending on the differences in the availability of habitat and prey. They also concluded that during the scarcity of food in a particular area, an owl might have to travel long distances to get adequate food that it needed to survive. It would hunt over a much smaller area if food abounds; the home ranges of neighbouring barn owls in food rich situation could overlap significantly (Jason *et al.,* 2005).

CONCLUSION

This study concludes that both the first and the second generation anticoagulant rodenticides led to a larger home range size of *T. alba.* The lack of adequate food source by the depletion of prey forced the owls to engage in greater exploratory flight. The implication would be the owls had to expand more energy to meet their metabolic requirements. For the females, this might have a drawback on its reproductive potential as it might offset the build-up of fat reserve for egg laying and incubation purposes. The application of biorodenticides had less impact on the ranging behaviour of the owls as the home range size was comparable to the control non-treated plot. The longer time to death, from biorodenticide bait consumption compared to anticoagulant rodenticides and the weaker infected rat, made them easier prey and thus less effort to secure sufficient prey. The host specific *S. singaporensis* has an added advantage compared to the anticoagulants since it poses no risk of secondary poisoning to the owl. Therefore, *S. singaporensis* would serve as a better choice rodenticide for a sustainable biological control of rats using *T. alba.*

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Systematic Significance of Leaf Anatomy in *Johannesteijsmannia* H.E. Moore (Arecaceae)

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ABSTRACT

A comparative leaf anatomy study was undertaken on *Johannesteijsmannia* H.E. Moore in Peninsular Malaysia in order to assess leaf anatomical variation which may be useful in species identification. All four species representatives of Malaysian Johannesteijsmannia were studied. Idioblast cells, sclereids, sclerenchymatous inner bundle sheaths, parenchymatous outer bundle sheaths, division of phloem in the vascular bundles, sub-epidermal fibrous strands below abaxial and adaxial epidermis are commonly present in all the species studied. The outline of the leaf margins, types of stomata, shaped of epidermis cells on the adaxial side, the position of vascular bundles in the leaf lamina and the presence of simple unicellular trichome, fibres at the tip of leaf margins, sub-epidermal fibrous strands in the mesophylls cells, hypodermis layer below adaxial epidermis and stegmata are useful in distinguishing individual species. In conclusion, anatomical characters have systematic significance in Johannesteijsmannia, especially at the species level.

Keywords: Systematic, leaf anatomy, *Johannesteijsmannia,* palms

INTRODUCTION

Johannesteijsmannia H.E. Moore is a genus in the tribe Corypheae Mart., subfamily Coryphoideae Griff. (Arecaceae), found in the tropical rainforests of southern Thailand, Malaysia and Indonesia (Uhl & Dransfield, 1987). The palms with huge, diamond-shaped fronds, known as daun payong, umbrella leaves, sal, sand, koh, lak, are one of the grand sights of the Malaysian forest (Lim & Whitmore, 2000; Tomlinson, 1990). Most of the *Johannesteijsmannia* species, especially *J. magnifica* and *J. altifrons,* are widely used in landscapes and popular as ornamental plants because of the uniqueness of their architecture. There are four species distributed within the Peninsula, of which the most widespread is *J. altifrons* (Rchb. f. and Zoll.) H. E. Moore, that is also native to south Thailand, north Sumatra, and Sarawak (Lim & Whitmore, 2000). The other three, *J. magnifica* J. Dransf.*, J. lanceolata* J. Dransf. and *J. perakensis* J. Dransf., are endemic to Peninsular Malaysia, and are relatively rare and highly endangered (Dransfield, 1972). The four species of the genus were previously assumed to be the same, originally named *Teysmannia altifrons*, discovered c. 1856 in Padang, Sumatra, by K.F. Stijman who sent it to J.E. Teysmann (also spelt *Teijsmann*). It was formally published in 1853 by Reichenbach and Zollinger, but in 1961, H.E. Moore Jr. changed the genus name to *Johannesteijmannia* H.E. Moore (Moore, 1961).

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The first attempt to study the anatomy of palm was by Solereder and Meyer (1928), however, the work suffered poor illustration. The most comprehensive study was conducted on *T. altifrons* by Tomlinson (1961). This article provides a guide for the leaf anatomical data on the four species of *Johannesteijsmannia* in detail. The overall objective of this study was to determine whether the leaf anatomical characters in *Johannesteijsmannia* could be of taxonomic value in systematic and diagnostic investigations, besides providing additional data on the leaf anatomical characters of this special genus. These anatomical characteristics would provide supporting evidence for the systematic treatment of the genus *Johannesteijsmannia.*

MATERIALS AND METHODS

Fresh specimens used in this study were obtained from various localities in Peninsular Malaysia, including the palm collection of Forest Research Institute, Malaysia, Kepong, Selangor, Gunung Angsi Forest Reserve, Negeri Sembilan and Kledang Sayong Forest Reserve, Perak, Gunung Bubu Forest Reserve, Perak and Semenyih Dam, Selangor. Three replicates were used to represent each species in this study. Voucher specimens of the freshly collected material were prepared by dry pressing, followed by mounting them onto the herbarium sheet, labelling and documenting. The voucher specimens were kept at Universiti Kebangsaan Malaysia Herbarium, Malaysia (UKMB) for future reference. A full list of the species studied and the localities from which they had been collected is given in Appendix 1. Fixation, embedding, sectioning, epidermal mechanical scrapping and stained were done according to the procedure by Johansen (1940) and Sass (1958), with suitable modifications. Fresh materials were fixed in AA (1:3) of acetic acid (30%) acetic acid and ethanol (70%). Dried herbarium materials were boiled, and fixed using the same solution. Leaf transverse section of the specimens were sectioned with a sliding microtome at 20 – 30 µm thickness and stained in 1 % Safranin in 50 % alcohol and 1 % Alcian Green in 100 ml purified water with three drops of acetic acid. Sections of the leaf marginal and lamina were made from the middle and marginal parts of the leaf lamina using a Reichert sliding microtome. All slides were mounted in Euparal after dehydration using alcohol series 50%, 70%, 95% and 100%. Photomicrographs of the sections were taken using either a Leitz Diaplan polarizing microscope fitted with a JVC CCD camera or Reichert Polyvar 2 Microscope fitted with a digital camera. These images were processed using Analysis Docu Software (soft-imaging system). All the slides were deposited in the anatomy section at Microtechnique Laboratory, Universiti Kebangsaan Malaysia, Malaysia.

RESULTS

Results showed some variations and similarities in the leaf anatomical characteristics of *Johannesteijsmannia* species studied. A summary of the leaf anatomical characteristics observed in this study is presented in Tables 1 and 2.

Epidermal cells anticlinal and periclinal walls: straight to curved anticlinal walls on the abaxial and adaxial epidermis of *J. altifrons, J. lanceolata, J. magnifica* and *J. perakensis* (*Fig. 1A – H*). **Stomatal complexes:** hypostomatic (present only on the abaxial epidermis); hexacytic in *J. altifrons* (*Fig. 2A*) and *J. perakensis* (*Fig. 2D*), tetracytic in *J. lanceolata* (*Fig. 2B*), octacytic in *J. magnifica* (*Fig. 2C*). **Trichomes:** present only on the abaxial epidermis of *J. magnifica*, simple multicellular trichomes (*Fig. 4M*). **Shaped of epidermal cells:** rectangular in *J. altifrons* (*Fig. 1E*), *J. lanceolata* (*Fig. 1F*) and *J. perakensis* (*Fig. 1H*), mostly hexagonal in *J. magnifica* (*Fig. 1G*). **Margin outline:** rounded in *J. magnifica* (*Fig. 4C*), pointed in *J. altifrons* (*Fig. 4A*), *J. lanceolata* (*Fig. 4B*) and *J. perakensis* (*Fig. 4D*). Meanwhile, fibrous cells were found to be present at the tip of the leaf margin of *J. lanceolata* (*Fig. 4B*), *J. magnifica* (*Fig. 4C*) and *J. perakensis* (*Fig. 4D*), but absent in *J. altifrons* (*Fig. 4A*). The presence of secondary and tertiary vascular bundles varies between the species; these are sparsely scattered

in *J. altifrons* and *J. lanceolata*, but densely scattered in *J. magnifica* and *J. perakensis*. **Leaf lamina:** adaxial and abaxial epidermal cells, 1 layer in *J. altifrons* (*Fig. 3A*), *J. lanceolata* (*Fig. 3B*), *J. perakensis* (*Fig. 3D*) and in *J. magnifica* (*Fig. 3C*). **Hypodermis**: one layer under adaxial epidermal cells, interrupted by sclerenchyma cells or fibrous strands or sub-epidermal fibres, was shown to be present in *J. altifrons* (*Fig. 3I*) and *J. magnifica* (*Fig. 3C*), and one layer of uniform hypodermis cells under adaxial epidermal cells in *J. lanceolata* (*Fig. 3B*) and *J. perakensis* (*Fig. 3D*). **Chlorenchyma:** palisade cells in 2 to 3 adaxial layers occupying ⅓ of leaf thickness, one cell to three times taller than the width. Spongy mesophyll cells in *ca* 6 – 8 layers are round, with no intercellular spaces. **Vascular bundles in the leaf lamina:** arranged in one row, approximately equidistant from the abaxial and adaxial epidermal layers in *J. lanceolata* (*Fig. 3B*) and *J. perakensis* (*Fig. 3D*), whereas these are close to abaxial epidermis in *J. altifrons* (*Fig. 3A*) and *J. magnifica* (*Fig. 3C*). Bundle sheaths, inner sheath; sclerenchyma cells completely ensheathing the vascular bundles, outer sheath-parenchyma cells in one layer incompletely ensheathing the vascular bundles (*Fig. 3A – H*). Phloem was divided into two sections by the layers of sclerenchyma cells that were present in all the species studied. **Sclerenchyma cells:** clusters of sclerenchyma cells or sub-epidermal fibres present under the adaxial and abaxial epidermis in all the species studied (*Fig. 3A – L*). Sub-epidermal fibres were also present between the mesophyll cells in *J. altifrons* (*Fig. 3A*), *J. lanceolata* (*Fig. 3B)* and *J. perakensis* (*Fig. 3D*), but absent in *J. magnifica* (*Fig. 3C*). **Cell inclusions:** silica bodies or stegmata alongside or close to the sub-epidermal fibres were present in *J. lanceolata* (*Fig. 5A*) and *J. perakensis*, but absent in *J. magnifica* and *J. altifrons;* idioblast cells present in the mesophyll cells very sparsely scattered in all the species studied, *J. altifrons* (*Fig. 5C* and *5I*); sclereids were found in spongy mesophyll or associated with fibrous vascular bundle sheaths in all the species studied, namely *J. magnifica* (*Fig. 5B*), *J. altifrons* (*Fig. 5C*), *J. perakensis* (*Fig. 5D*) and *J. lanceolata* (*Fig. 5E*). **Trichomes:** simple unicellular were shown to be very sparsely scattered and present only on the abaxial epidermis of *J. magnifica* (*Fig. 4K*) but absent in *J. altifrons, J. lanceolata* and *J. perakensis.*

DISCUSSION AND TAXONOMIC IMPLICATIONS

Lamina Transverse Sections

Tomlinson (1961) reported the presence of one-layered hypodermal cells below the abaxial and adaxial epidermal cells in some Arecaceae species. Cell files, which are often replaced by fibres and cells, are normally small, more or less cubical, and slightly thick-walled. The hypodermis layer is present in all the four species studied. In *J. altifrons* and *J. magnifica*, they are similar with one-layered below the adaxial epidermal cells, but interrupted with non-vascular fibres. It appears that *J. lanceolata* and *J. perakensis* have a uniform hypodermis layer. The cells are more or less cubical, slightly thick walled with two or three small sclerotic cells surrounding each sub-stomatal chamber. In the previous study in *Licuala*, one layer of hypodermal cells below the abaxial and adaxial epidermal cells were observed (Tomlinson, 1961). Adaxial cells are more or less cubical, most conspicuous and uniform. Hypodermal cells present in the abaxial epidermal cells are less regular and smaller. The result of this study has shown that the presence of hypodermis layer can be a diagnostic character for *Johannesteijsmannia*, and thus, it has taxonomic significance.

Veins are rather remote from each other, more or less equidistant and always separated from the epidermis by spongy and palisade mesophyll. The position of vascular bundles in leaf lamina is approximately equidistant from the abaxial and adaxial epidermal layers in the two species (*J. lanceolata* and *J. perakensis*), but close to abaxial epidermis in the other two spexies (*J. altifrons* and *J. magnifica*). All the *Johannesteijsmannia* species possess vascular bundles which are completely ensheathed

TABLE 1
Leaf lamina and margin anatomical characteristics in *Johannesteijsmannia* species

Species	Leaf lamina							Leaf margin		
		Vascular bundles			Sub-epidermal fibres					
	Hypodermis	Location	Parenchymatous sheath Schlerenchymatous sheath	Stegmata	Below adaxial and abaxial epidermis	In the mesophyll cells	Idioblast and sclereids cells	Marginal fibre strands	Outline	The presence of secondary and tertiary vascular bundles
J. altifrons	1 layer interrupted by non-vascular fibres	Closed to the abaxial epidermis	Present	Absent	Present	Present	Present	Absent	Pointed	Sparsely scattered
J. lanceolata	Uniform	Equidistant from the abaxial and adaxial epidermis	Present	Present	Present	Present	Present	Present 'V' shaped	Pointed	Sparsely scattered
J. magnifica	1 layer interrupted by non-vascular fibres	Closed to the abaxial epidermis	Present	Absent	Present	Absent	Present	Present 'U' shaped	Rounded	Densely scattered
J. perakensis	Uniform	Equidistant from the abaxial and adaxial epidermis	Present	Present	Present	Present	Present	Present Arc shaped	Pointed	Densely scattered

TABLE 2
Leaf surface anatomical characteristics in *Johannesteijsmannia* species

Species	Stomata	Trichomes	Anticlinal walls		Shaped of epidermis cells	
			Adaxial epidermis	Abaxial epidermis	Adaxial epidermis	Abaxial epidermis
J. altifrons	Hexacytic	Absent	Straight to curved	Straight to curved	Cell rectangular	Cell rectangular
J. lanceolata	Tetracytic	Absent	Straight to curved	Straight to curved	Cell rectangular	Cell rectangular
J. magnifica	Octacytic	Simple multicellular	Straight to curved	Straight to curved	Cell hexagonal	Cell rectangular
J. perakensis	Hexacytic	Absent	Straight to curved	Straight to curved	Cell rectangular	Cell rectangular

by sclerenchyma cells (as an inner sheath) and incompletely ensheathed by a layer of parenchyma cells (as an outer sheath). This study has shown that sclerenchyma cells that completely surrounded the vascular bundles and parenchyma cells that incompletely surrounded the vascular bundles in the lamina are common characters for all the *Johannesteijsmannia* species undertaken in the study. These characters are also common in most palms species (Tomlinson, 1961; Passos & De Mendonca, 2006), and therefore have less systematic value. The phloem tissues in the main vascular bundles of all the *Johannesteijsmannia* species studied have characteristics form with its phloem divided into two separating sections. This character is also found in some other palms species such as in *Licuala rumphii, Mauritia flexuosa* and *Raphia vinifera* (Tomlinson, 1961); therefore, this character also has less systematic value for distinguishing species in *Johannesteijsmannia*, yet taxonomically significant for the species in Areacaceae.

The outline of the transverse sections of the margin is pointed or rounded, pointed in *J. altifrons, J. lanceolata* and *J. perakensis*, but rounded in *J. magnifica*. Thus, it is certainly a diagnostic character for this particular species. The leaf margin is not frequently used in systematic study. However, Talip *et al.* (2005) stated that this character is useful in the identification of some *Zingiberaceae* species. This anatomical characteristic definitely has systematic significance in *Johannesteijsmannia,* especially in the presence of fibrous cells or strands at the tip margin. The fibrous cells were found to be present in *J. lanceolata, J. magnifica* and *J. perakensis* but absent in *J. altifrons*. The presence of marginal fibrous strands or cells in *J. lanceolata, J. magnifica* and *J. perakensis* has characteristic outlines. In this study, it was shown that *J. lanceolata* has marginal fibre strands shaped No. 3 ('V' shaped), while *J. magnifica* has marginal fibre strands shaped No. 1 (U shaped) and *J. perakensis* has marginal fibre strands shaped No. 2 (arc shaped). Wu and Cutler (1985) also stated that the presence of marginal fibrous strands is constant, reliable diagnostically and taxonomically useful characters in Iris.

Cell Inclusions

Silica is most common in palms as small bodies and these silica cells are the stegmata named by German authors (Tomlinson, 1961). Stegmata are usually the most abundant adjacent to the vascular and fibrous bundles of the stem and leaf (Dickison, 2000). In this study, stegmata present in the mesophyll cells in long continuous files, adjacent to the abaxial fibrous strands in only two species, namely, *J. lanceolata* and *J. perakensis*. The silica bodies are spherical, which are rather irregular, more symmetrical and druse-like. The presence of stegmata in this study could therefore be very useful in distinguishing some species in this genus.

The presence of the sclereids and idioblast cells in the leaf lamina in all the species studied is a common anatomical character of this particular genus. Sclereids typically are short cells with thick secondary walls, strongly lignified and provided with numerous simple pits (Evert *et al.,* 2006). Leaves are an especially rich source of sclereids; as for the variety of form, however, they are rare in the leaves of monocots (Tomlinson, 1959, 1961). The presence of sclereids is uncommon in palm leaves, apart from sclerotic mesophyll cells in some species in *Eugeissona, Licuala, Daemonorops* and *Lodoicea* (Tomlinson, 1959, 1961). The foliar sclereids found in *Eugeissona* are short, lignified and columnar, but long, unlignified and fibre-like in *Bactris* and *Licuala* (Tomlinson, 1959). In *Daemonorops,* fibre-like sclereids are present, and this can be homologous with the fibres ensheathing the tranverse veins. In this study, the foliar sclereids found to be present in mesophyll cells or attached to the vascular bundle sheaths. The sclereids were shown to be filiform, lignified and columnar, but long and present in all the *Johannesteijsmannia* species studied. Although sclereids in palms have no obvious biological function, they are quite useful in diagnosis especially at generic level.

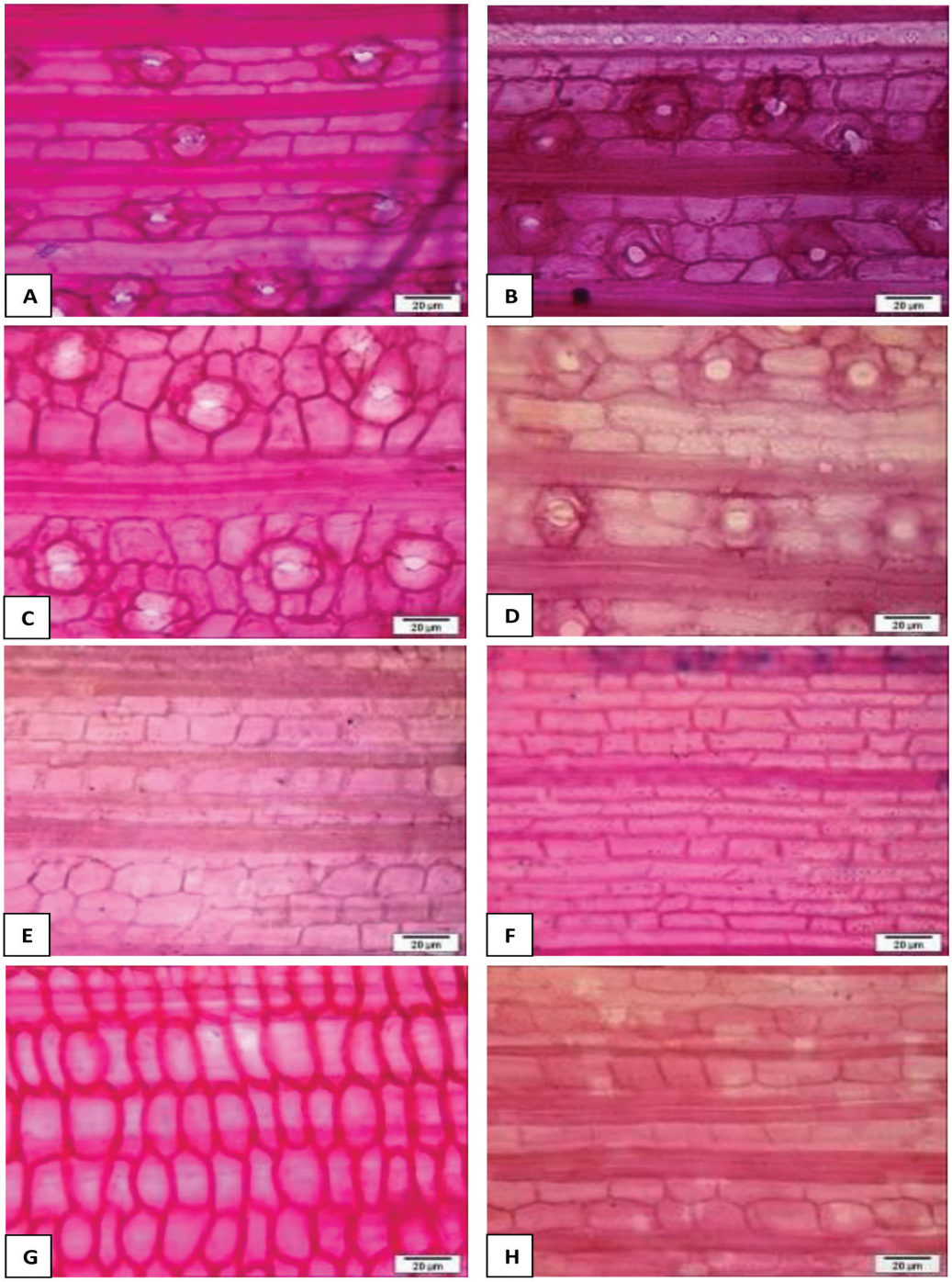

Fig. 1A-H: Leaf surface of epidermis in Johannesteijsmannia *species. Abaxial side: A – D), (A)* J. altiforns*, (B)* J. lanceolata*, (C)* J. magnifica*, and D)* J. perakensis*. Adaxial side: E – H), (E)* J. altifrons*, (F)* J. lanceolata*, (G)* J. magfinica*, and (H)* J. perakensis*. Scale bar 20 µm*

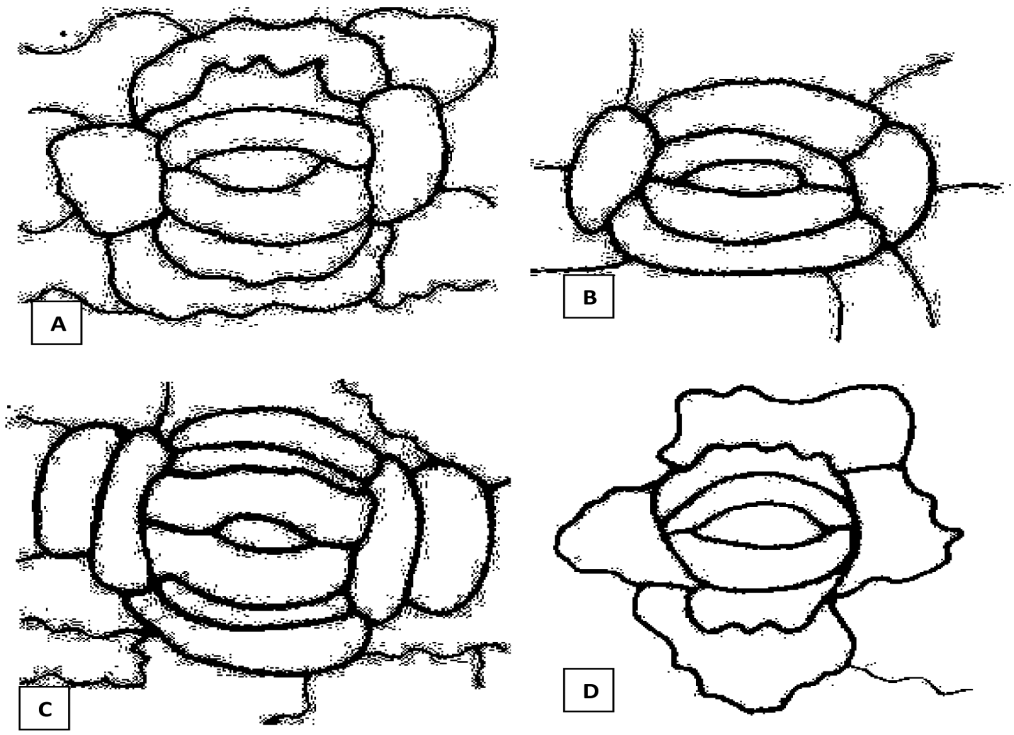

Fig. 2A - D: Types of stomata in Johannesteijsmannia *species; (A)* J. altifrons *(hexacytic), (B)* J. lanceolata*, (tetracytic), (C)* J. magnifica *(octacytic), and (D)* J. perakensis *(hexacytic). Scale bar: 20 µm*

Leaf Epidermis

Leaf epidermal characteristics are of taxonomical importance in many plants species (Stace, 1965, 1969; Wilkinson, 1979; Baranova, 1972, 1987, 1992a,b, 2004; Barthlott *et al*., 1998; Barthlott, 1981, 1990; Kong, 2001; Carpenter, 2005). The finding in this study has shown that this characteristic is of taxonomic value in the genus *Johannesteijsmannia*. The anticlinal wall of the adaxial and abaxial epidermal cells is straight to curvy in all the species studied, and appears to be not a good anatomical characteristic to differentiate the species. In the adaxial epidermal cells of *J. altifrons, J. lanceolata* and *J. perakensis,* the cuticular deposit has sinuous appearance, and this sinuous appearance of cuticular deposit occurs on the abaxial epidermal cells of all the species studied at a high level of focus, whilst at a low level, the wall is not sinuous. This characteristic was reported by Tomlinson (1961) in other palms genera. However, there are many palm species with sinuous anticlinal walls, such as in *Mauritia flexuosa* which can be a characteristic of this species within the genus (Passos & De Mendonca 2006).

In the abaxial epidermis above the hypodermal fibrous strands, there are files of long narrow cells (costal bands) which alternate with the files of wider cells (intercostals bands) elsewhere. Stomata are situated in the intercostals band and the files of the cells in the intercostals bands are quite similar between the species, and this is specifically 2 to 3 in *J. lanceolata* and *J. magnifica*, 2 to 4 in *J. perakensis* and with 3 to 5 files of the cells in *J. altifrons*. In the adaxial epidermal, there are also files of costal bands cells, cell rectangular,

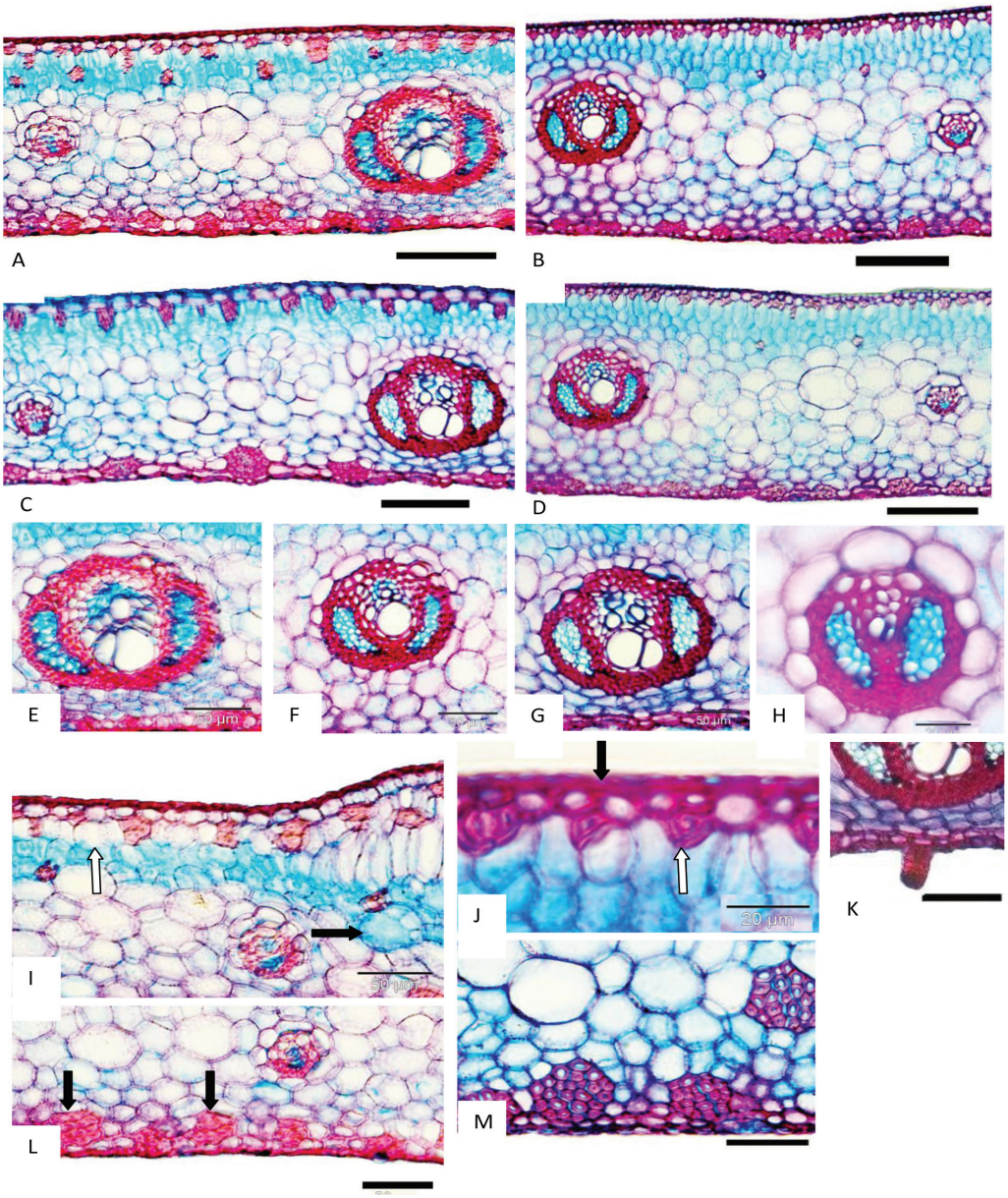

Fig. 3A-L: Leaf lamina transverse sections; (A) J. altifrons*, (B)* J. lanceolata*, (C)* J. magnifica*, (D)* J. perakensis*. Vascular bundles; (E)* J. altifrons*, (F)* J. lanceolata*, (G)* J. magnifica*, (H)* J. perakensis*, (I)* J. altifrons*, an idioblast cell (black arrow) and an interrupted hypodermis layer (white arrow), (J)* J. lanceolata*, sub-epidermal fibrous cells (white arrow) and cutinized epidermal cells (black arrow), (K)* J. magnifica*, simple multicellular trichome, (L)* J. magnifica*, sub-epidermal fibrous cells below the abaxial epidermis (black arrow), and (M)* J. perakensis*, sub-epidermal fibrous cells below the abaxial epidermis. Scale bar: (A – D) =100µm, E – I & K) = 50 µm, J, L & M) = 20 µm*

longitudinally extended, elongated parallel to the long axis of the leaf segment or leaflet, and some of the cells are almost cubical in all species studied. Nevertheless, the cells are hexagonal in shape in *J. magnifica.* The findings in this study have shown that the leaf epidermal characteristics can be used in distinguishing some species; therefore, this characteristic has its systematic significant in this genus.

Stomata

According to Solereder (1908), Metcalfe and Chalk (1950), Tomlinson (1961), Stace (1965) and Rajagopal (1979), the distribution patterns of stomata based on their orientation and dispersion are found to be fairly stable and could therefore be taxonomically useful. Stomata are restricted to abaxial intercostal regions and not in distinct or costal files of cells. The pattern of the stomata in this present study was classified as scattered and the stomata are often remote from each other. This result confirmed the observation made by Tomlinson (1961).

The common type of stomata in the palm species is paracytic (Tomlinson, 1961), or tetracytic (Tomlinson, 1961; Pérez & Rebollar, 2003). Tomlinson (1961) reported the type of stomata in this genus as being tetracytic and paracytic, while in this study, hexacytic stomata was found in *J. altifrons* and *J. perakensis*, tetracytic in *J. lanceolata* and octacytic in *J. magnifica.* In this study, it is therefore evident that the types of stomatal complexes have a significant taxonomic value in this genus, which can be used to differentiate between the four species.

CONCLUSIONS

Some of the anatomical characteristics observed in this study revealed a number of interesting

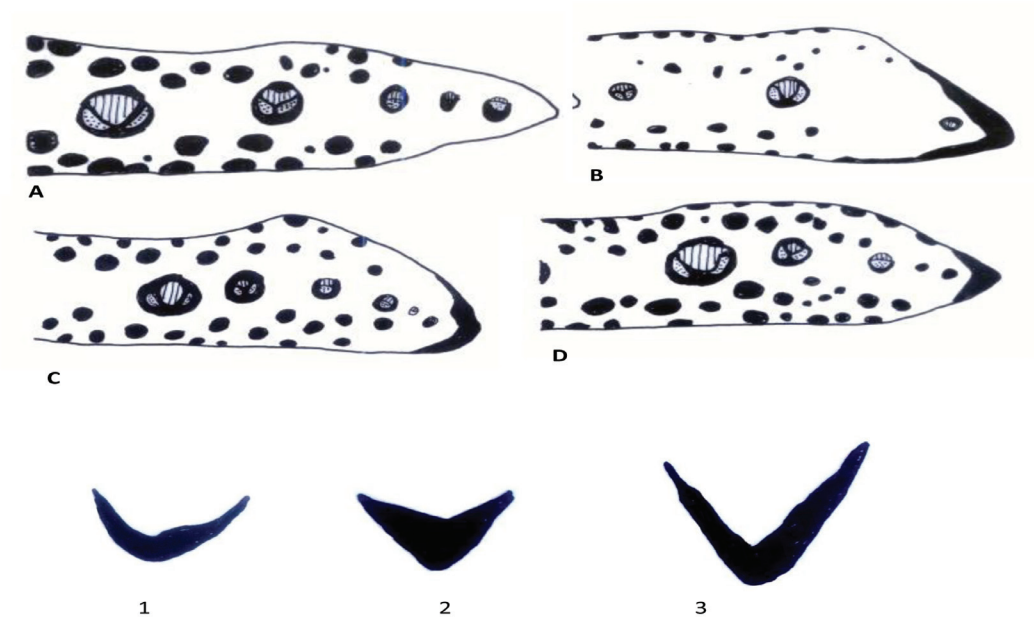

Fig. 4: Transverse sections of the leaf margins in Johannesteijsmannia *species indicating margin shape and the presence of marginal fibre strands. (A)* J. altifrons *(pointed), (B)* J. lanceolata *(pointed), (C)* J. magnifica *(rounded), (D)* J. perakensis *(pointed). 1 = Marginal fibrous strands 'U' shaped (*J. magnifica*), 2 = Marginal fibrous strands arc shaped (*J. perakensis*), and 3 = Marginal fibrous strands 'V' shaped (*J. lanceolata*). Scale bar: (A- D) = 50 µm*

features, with some characters which can be of taxonomic and diagnostic values. For example, sclereids and idioblast cells are common anatomical characters in the genus indicating close interrelationship between the species. The results of this study have shown that the leaf anatomical evidence can be used for identifying certain species in *Johannesteijsmannia*, such as in the presence of marginal fibre strands in all the species, except in *J. altifrons*, as the outline of the margin pointed in *J. altifrons, J. lanceolata* and *J. perakensis* but rounded in *J. magnifica,* Those characteristics provide some additional anatomical data of this particular genus. Meanwhile, the anatomical characters observed in this study revealed a number of interesting features with some characteristics which could be of taxonomic and diagnostic values. The features of the stomata may be diagnostic in *J. lanceolata* with tetracytic stomata and *J. magnifica* with octacytic stomata. Thus, the anatomical data obtained from the present study are useful to construct the artificial identification key to the species in *Johannesteijsmannia*.

Key to identification of four *Johannesteijsmannia* species using leaf anatomical characters:

1 Hypodermis layer uniform; stegmata present; vascular bundles equidistant from the abaxial and adaxial epidermis 2

1 Hypodermis 1 layer interrupted by non-vascular fibres; stegmata absent; vascular bundles closed to the abaxial epidermis ……3

2 Marginal fibrous strands 'V' shaped; stomata tetracytic.….*J. lanceolata*

2 Marginal fibrous strands arc shaped; stomata hexacytic…….*J. perakensis*

3 Sub-epidermal fibres present in the mesophyll cells; marginal fibres strand absent in the leaf margin; shape of the abaxial epidermis cell – rectangular; leaf margin outline rounded; glabrous..... *J. altifrons*

3 Sub-epidermal fibres absent in the mesophyll cells; marginal fibres strand present in the leaf margin - arc shaped; shape of the adaxial epidermis cell – hexagonal; leaf margin outline pointed; simple multicellular trichomes.…….*J. magnifica*

Therefore, the anatomical features of this particular genus have been proven to be useful in the identification of species and they definitely have taxonomic values, specifically at the species level.

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APPENDIX 1
Details of the specimens of *Johannesteijsmannia* species examined

No	Species	Code	Locality	Collectors and date of collection
	J. altifrons	NT 100	FRIM Kepong, Selangor	Noraini Talip Mohd. Ruzi Abdul Rahman Nurul Hajar Razman 03.08.07
		NT 215	Hutan Simpan Endau Rompin, Johor	Noraini Talip Mohd. Ruzi Abdul Rahman 13.11.07
		NT216	Hutan Simpan Endau Rompin, Johor	Noraini Talip Mohd. Ruzi Abdul Rahman 13.11.07
	J. lanceolata	NT 97	FRIM Kepong, Selangor *Ex-situ* collection	Noraini Talip Mohd. Ruzi Abdul Rahman Nurul Hajar Razman 03.08.07
		NT230	Gunung Angsi, Negeri Sembilan	Noraini Talip Mohd. Ruzi Abdul Rahman 19.02.08
		RZ31	Gunung Angsi, Negeri Sembilan	Mohd. Ruzi Abdul Rahman 19.02.08
	J. magnifica	NT 99	FRIM Kepong, Selangor *Ex-situ* collection	Noraini Talip Mohd. Ruzi Abdul Rahman Nurul Hajar Razman 03.08.07
		NT232	Gunung Angsi, Negeri Sembilan	Noraini Talip Mohd. Ruzi Abdul Rahman 19.02.08
		NT244	Gunung Angsi, Negeri Sembilan	Noraini Talip Mohd. Ruzi Abdul Rahman 19.02.08
		RZ30	Empangan Semenyih, Selangor	Mohd. Ruzi Abdul Rahman 10.11.08
	J. perakensis	NT239	Kledang Saiong, Perak	Noraini Talip Mohd. Ruzi Abdul Rahman 21.02.08
		NT243	Kledang Saiong, Perak	Noraini Talip Mohd. Ruzi Abdul Rahman 21.02.08
		RZ33	Hutan Simpan Gunong Bubu, Perak	Mohd. Ruzi Abdul Rahman 10.10.08

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Genetic Relationships among Soybean Accessions Based on Morphological and RAPDs Techniques

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ABSTRACT

Morphological and molecular characterization of forty soybean (*Glycine max* (L.) Merr.) accessions were assessed using 7 morphological characters and 10 Randomly Amplified DNA Polymorphism (RAPD) primers, respectively. The field experiment was carried out at two locations using randomized complete block design with three replications. The aims of the research were to analyze the morphological and molecular organization of the existing diversity and to compare the genetic relatedness among forty soybean genotypes. The data were subjected to analysis of variance and correlation analysis to determine the extent of genetic variability and correlation coefficients among the characters, respectively. Principal Component Analysis (PCA) and Single Linkage Cluster Analysis (SLCA) were employed to group the accessions. The genetic relatedness among the accessions based on RAPD molecular markers was also presented in the form of a dendrogram generated by cluster analysis using the Unweighted Pair Group method with Arithmetic Mean (UPGMA). The relative effectiveness of the RAPD markers compared to botanical descriptors in assessing the diversity among the genotypes was investigated. The single linkage cluster technique classified the 40 accessions into seven clusters while the FASTCLUS technique revealed that the number of pods per plant, pod yield per plant, 100-seed weight and seed yield per plot contributed the largest proportion of morphological variation. Out of the 100 bands generated by the 10 primers, 31 were monomorphic and 69 polymorphic. The size of the fragment varied from 250 bp to 3000 bp. RAPDs markers were highly polymorphic and more discriminatory and informative as they were able to differentiate more pairs of genotypes than the botanical descriptors. The highest yield was recorded for TGx 1834-1E (477.60g/plot) and TGx 1910-2F (459.55).

Keyword: Genotypes, morphology, polymorphism, RAPD, soybean, UPGMA

INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is a principal grain legume in developing countries where it meets the expanding needs for protein, edible oil and calories. It is a good source of cheap dietary protein in Africa (IITA, 1989). It is a miraculous crop due to its extraordinary qualities; it contains about 37-42% good quality protein, 6% ash, 29% carbohydrate and 17-24% oil, comprising 85% poly-unsaturated fatty acid with two essential fatty acids (lenoleic and linolenic acid), which are not synthesized by the human body (Antalina, 2000; Balasubramaniyan & Palaniappan, 2003). Soybean is grown in the tropical, subtropical and temperate climates.

A number of tropical soybean varieties, with improved yield and agronomic characteristics, have been developed and recommended to farmers (FAO, 1999). However, the selection and subsequent recommendation for release

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of most of these varieties have been based on a subjective traditional analysis of the yield data from a number of locations, with little or no emphasis on morphological and molecular characterizations.

The assessment of the genetic relationships among the cultivated plants at morphological and molecular levels is a fundamental component of crop improvement programmes. This will provide information about genetic diversity, identification of diverse parental combinations to develop segregating progenies with maximum genetic variability for further selection, duplication in germplasm, and introgression of desirable genes or chromosome segments from diverse sources into elite germplasm (Thompson *et al*., 1998; Das *et al*., 2001; Iqbal *et al*., 2008).

Diversity, based on the morphological characters, is essential in plant breeding as it reveals important traits to plant breeders (Singh, 1989); however, the phenotypes are highly influenced by many environmental factors. In plants with a narrow genetic base in their gene pool, such as soybean, molecular descriptors can provide additional information about the characterization, degree of diversity and genetic constitution of the existing germplasm.

Currently, the rapid development of biotechnology allows easy analysis of large numbers of loci distributed throughout the genome of plants (Chakravanthi & Naravaneni, 2006). The use of RAPD technique lies in its simplicity, rapidity, requirement for only a small quantity of DNA and the ability to generate numerous polymorphisms (Chowdhury *et al*., 2001; Zenglu & Nelson, 2002; Yu *et al*., 2005). Therefore, it has been a powerful and useful technique for genetic analysis. The objectives of this study were to investigate the morphological and molecular organizations of the existing diversity in forty soybean accessions and to ascertain the genetic relatedness among the accessions.

MATERIALS AND METHODS

The forty accessions used in the study were collected from the Genetic Resources Unit of the International Institute of Tropical agriculture (IITA), Nigeria. The experimental study was conducted during the wet season of the year 2006 at two locations, namely, Abeokuta (longitude: 07^0 30'N and latitude: 03^0 54'E) and Ile-Ife (longitude: 07^o28'N and latitude: 03^o34'N), Nigeria. The randomized complete block design, with three replications, was used for the experiment. Seeds were sown by drilling in four-row plots at 0.75m between rows. Two weeks after planting, seedlings were thinned down to a within-row spacing of 0.05 m leaving a population of 480 plants per plot size of $18m^2$. No pre-emergence herbicides and fertilizer was used and weeding was carried out as required.

Morphological and physiological data were collected on ten plants per accession from the net plot for days to 50% flowering, days to maturity, number of pods per plant, pod length per plant, pod yield per plant, seed yield per plant and 100-seed weight. The data collected were subjected to statistical analysis using SAS/PC version 9. The principal component grouping of the traits was employed to examine the percentage contribution of each trait to the total genetic variation. Meanwhile, cluster analysis based on similarity matrices was also employed on agro-botanical data using the un-weighted pair group method with arithmetic mean (UPGMA) to obtain a dendrogram. The cultivars were sorted into groups by the FASTCLUS procedure of the SAS.

RAPD Analysis

Total DNA was isolated from the fresh leaves of 14 day-old soybean seedlings, grown in the green house, according to Dellaporta *et al*. (1983). Gene-based RAPD analysis was performed on forty soybean accessions. Purified DNA was quantified by spectrophotometry and by ethidium bromide staining after electrophoresis. Ten RAPD primers (Table 6) were used to generate markers as described by Tao *et al*. (1993). Each amplification was performed in a reaction volume of 25µl containing 10mM Tris-HCl pH 9.0, 50mM $MgCl_2$, 0.2mg mL^{-1} gelatine, Triton x 100.0%, 0.1mM of each of dATP, dCTP,

dGTP and dTTP (Promega), 10ng of random primer, 50ng of genomic DNA and 2 units of Taq polymerase. Amplification was carried out in a thermocycler (Mpi model), as follows: one cycle of 3mins at 94°C, 44 cycles of 20 secs at 94°C; 40 secs at 37°C and 1 min at 72°C; one cycle of 7 mins at 72°C. The amplification products were then analyzed for polymorphism after electrophoresis in 1.4% agarose gels using 2.8g in 200mls 1 X TAE buffler Pairwise comparison of genotypes, based on the presence (1) or absence (0) of unique and shared polymorphic products used to generate similarity coefficients using the statistical software NTSYS-pc 2.0 (Rohlf, 1993). GSij = Nij / (Ni + Nj- Nij), where Ni is the number of detected bands in a variety i and not in variety j, Nj is the number of detected bands in a variety j and not in variety i, and Nij is the number of bands common to the varieties i and j (Jaccard, 1908). The similarity coefficient was used to construct a dendrogram by the UPGMA, according to Sneath and Sokal (1973), Swofford and Olsen (1990) and Rohlf (1993).

RESULTS

The combined analyses of variance for days to 50% flowering, days to maturity, number of pods per plant, pod length per plant, pod yield per plant, seed yield per plot and 100-seed weight revealed highly significant ($P < 0.01$) genotypic variation for all the traits (Table 1). The effect due to location was also highly significant for all the traits. Genotype x location effects were not significant for all the traits, except for the seed yield per plot. The seed yield per plot varied from one location to another, indicating that the selection for yield per plot has to be done at each location.

Table 2 presents the mean performance of 40 soybean accessions sown over two locations. TGM 80 and TGx 1830-20E recorded the earliest flowering day (37 days) and earliest maturity day (82 days), while TGM 119 recorded the longest flowering day (47 days) and the longest maturity day (93 days). TGx 1834-1F with the highest pod yield per plant of 6.7g had 100-seed weight of 11.1g whereas TGx 1919-8F, with pod yield per plant of 4.8g had the highest 100-seed weight of 18.0g. In addition, TGM 1906-1F had the highest number of pods (23) per plant.

Table 3 shows the correlation coefficients of the pairs of six traits that were used in characterizing the forty soybean accessions. The correlation matrix showed that seed yield per plot was positively and significantly associated with number of pods per plant, pod length, pod yield per plant and 100-seed weight. Positive and significant association was also observed between the number of pods per plant, pod length, pod yield per plant and 100-seed weight. Meanwhile, days to 50% flowering and Days to maturity were both negatively associated with 100-seed weight and seed yield per plot.

The three principal components accounted for 82.7% of the total variance, with the first principal component taking 51.24%. The relative Eigen values was high (3.66) for axis 1 and low (0.72) for axis 3. The first principal component was mostly correlated with the number of pods per plant, pod length, pod yield per plant, 100-seed weight and seed yield per plot. The characters that were mostly correlated with the second principal component were days to 50% flowering and days to maturity (Table 4).

The characteristic means of the seven similarity cluster groups in the 40 soybean accessions generated by the FASTCLUS technique are presented in Table 5. An examination of the range of means revealed that the number of pods per plant, pod yield per plant, 100-seed weight and seed yield per plot contributed the largest proportion of morphological variations that existed between the cluster groups. Nonetheless, there was no significant variation between clusters with respect to other traits. Cluster V consisted of 2 accessions, which represented 5% of the total accessions. The maximum number of pods per plant (19.96), pod yield per plant (6.57g), 100-seed weight (13.13g) and seed yield per plot (468.58g) were found in cluster V. Cluster IV accounted for 2.5% of the population. This cluster showed the least value for the number

TABLE 1
Combined mean squares of seed yield and related traits for soybean accessions sown in two environments

Source	df	Seed yield per plot (g)	Pod yield/ plant (g)	Days to 50% flowering	Days to maturity	Number of pods/plant	Pod length (cm)	100 seed weight (g)
Block	2	2.51**	6.90**	6.02	2.30	23.01**	0.59	10.79
Genotypes (G)	39	2.33**	4.97**	18.26**	5.27**	39.73**	0.91**	22.90**
Location (L)	1	126.72**	81.28**	403.00**	250.10**	2247.05**	29.19**	320.31**
G x L	39	0.86*	1.14	4.26	2.42	6.16	0.38	2.71
Error	158	0.54	1.29	3.87	1.70	4.29	0.21	7.01

*, ** Significant at 5% and 1% probability levels, respectively

TABLE 2
Mean performance of 40 soybean accessions sown in two locations in 2006

S/No	Accession	Seed yield/plot (g)	Pod yield/plant (g)	Days to 50% flowering	Days to maturity	Number of pods/plant	Pod length (cm)	100 seed weight (g)
1	TGx1909-3F	443.33	6.0	43	88.5	17	4.5	12.0
2	TGx 1904-6F	379.75	5.4	42	87.0	18	4.8	9.6
3	TGx 536-02D	302.25	4.3	44	88.5	15	4.3	8.3
4	TGx 1740-2F	359.03	5.6	43	87.0	18	4.5	10.3
5	TGx 1485-1D	354.35	5.2	42	87.0	15	4.2	10.1
6	TGx 1440-1E	336.25	4.9	42	86.0	19	4.2	8.9
7	TGx 1835-10E	348.05	4.8	42	86.5	15	4.9	9.3
8	TGx 1866-2F	433.00	6.4	43	88.5	18	4.4	12.8
9	TGM 119	252.80	3.4	47	92.5	16	3.6	8.6
10	UG-5	235.05	3.1	42	85.0	13	3.6	7.3
11	TGx 1904-4F	329.70	4.4	42	85.5	15	3.7	10.9
12	TGx 1910-14F	268.25	4.0	43	87.0	13	4.2	11.3
13	TGx 1883-33F	385.78	5.3	43	86.0	16	4.4	11.4
14	TGx 1908-8F	337.00	4.5	42	85.5	16	4.4	10.6

Table 2 (*continued*)

S/No	Accession	Seed yield/plot (g)	Pod yield/plant (g)	Days to 50% flowering	Days to maturity	Number of pods/plant	Pod length (cm)	100 seed weight (g)
15	TGx 1903-5F	322.87	4.0	43	87.0	14	4.2	11.1
16	TGx 1912-13F	301.65	4.5	44	87.5	14	4.4	12.1
17	TGM 63	252.05	3.7	43	87.0	13	4.0	9.9
18	TGM 255	211.02	3.5	42	86.5	13	3.4	9.1
19	TGx 1908-1F	395.35	5.3	40	84.0	16	4.4	11.7
20	TGx 1906-1F	290.00	4.0	42	87.5	13	3.8	11.8
21	TGM 479	441.15	5.3	43	87.0	23	4.4	13.2
22	Samsory-2	352.53	5.2	43	87.5	14	4.5	11.3
23	TGx 1910-3F	425.73	5.7	45	90.0	16	4.1	11.4
24	TGx 1878-7E	326.50	4.3	43	87.0	17	4.1	12.2
25	TGx 1884-18E	395.55	5.6	42	86.5	16	4.6	12.2
26	TGx 1910-2F	459.55	6.5	42	83.5	19	4.8	14.5
27	TGx 1834-1E	477.60	6.7	44	86.5	21	5.0	11.8
28	TGx 1919-8F	343.55	4.8	44	88.0	15	4.1	18.0
29	TGx 1908-9F	447.00	6.2	43	87.0	21	4.7	14.8
30	TGx 1448-2E	344.30	4.9	43	85.0	15	4.4	11.9
31	TGx 1830-20E	419.30	5.8	37	81.5	20	4.8	11.9
32	TGx 1838-5E	417.05	5.4	39	83.0	17	4.5	11.7
33	TGx 1888-15F	292.25	3.7	42	87.5	15	3.5	9.9
34	TGx 1844-4E	365.38	4.7	43	88.5	16	4.6	11.4
35	TGM 79	359.65	5.0	43	88.5	21	4.9	11.7
36	TGx 1903-3F	409.05	6.0	43	88.5	18	4.7	13.2
37	TGM 80	360.55	4.4	37	82.0	17	4.0	9.9
38	TGx 1019-2EB	293.73	4.0	42	86.5	15	4.2	13.9
39	TGM 1197	435.00	5.4	43	86.5	20	5.0	11.6
40	TGx 1902-1E	428.05	6.1	43	87.5	19	4.4	13.0
	LSD	82.80	1.29	1.85	1.28	2.36	0.52	2.99

TABLE 3
Correlation coefficients of six traits used in characterizing 40 soybean accessions

	Days to maturity	Number of pods per plant	Pod length (cm)	Pod yield per plant (g)	100-seed weight (g)	Seed yield per plot (g)
Days to 50% flowering	0.72**	-0.20**	-0.31**	-0.23**	-0.17**	-0.37**
Days to maturity		-0.14**	-0.23**	-0.22**	-0.18**	-0.34**
Number of pods per plant			0.54**	0.68**	0.40**	0.68**
Pod length				0.63**	0.39**	0.65**
Pod yield per plant					0.52**	0.87**
100-seed weight						0.55**

** Significant at 1% probability levels

TABLE 4
Principal components analysis showing the contribution (factor scores) of each character among the 40 soybean genotypes, Eigen values and percentage total variance accounted for by four principal components

Character	Prin 1	Prin 2	Prin 3	Prin 4
Days to 50% flowering	-0.11	0.69	-0.04	0.11
Days to maturity	-0.13	0.68	-0.15	-0.06
Number of pods per plant	0.44	0.07	-0.26	-0.67
Pod length	0.44	0.02	-0.27	0.72
Pod yield per plant	0.49	0.11	-0.06	0.03
100-seed weight	0.30	0.17	0.91	0.01
Seed yield per plot	0.50	0.05	-0.07	-0.07
Eigen value	3.66	1.85	0.72	0.38
% variance	0.52	0.26	0.10	0.05
Cumulative % variance	0.52	0.79	0.89	0.94

of pods per plant (12.42), pod yield per plant (3.52), 100-seed weight (9.09) and seed yield per plot (211.02).

The ten primers were assessed for their consistent production of strong amplification and reproducible bands across forty soybean genotypes (Table 6). A total of 100 bands were generated using ten selected primers. The number of polymorphic bands produced by each primer varied from four (OPG 11) to ten (OPG 16). Out of the 100 bands observed, 31 were monomorphic for all the varieties examined in this study. The remaining 69 variable bands were reproducible and polymorphic and thus, they were regarded as informative RAPD markers for the current genetic study as these markers were able to differentiate all the accessions. Each accession could be distinguished by at least four RAPD markers. The size of each fragment varied from 250 bp to 3000 bp, depending on the primer used for the amplification.

Fig. 1 shows the morphological dendrogram. It reveals the minimum distance between the clusters and the extent of morphological relationships between pairs of genotypes within each cluster group. The diagram proposed seven groups. TGx 1740-2F (4) in group 1; TGx 1904-6G (2), TGx 1440-1E (6), TGx 1844-4E (34) and

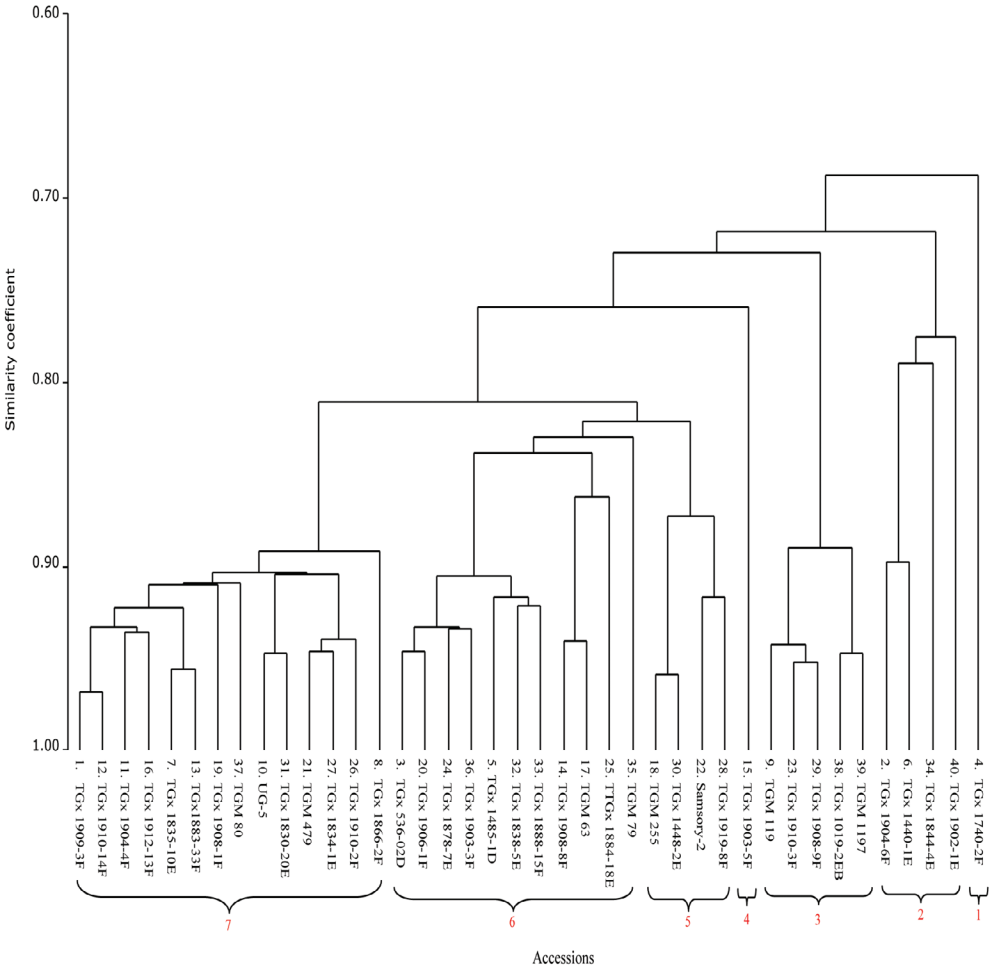

Fig. 1: Morphological dendrogram showing the similarity among 40 soybean accessions revealed by UPGMA cluster analysis

TGx 1902-1E (40) in group 2; TGM 119 (9) and TGM 197 (39) in group 3; TGx 1908-8F (14) and TGM 63 (17) in group 4; TGM 255 (18), Samsoy-2 (22), TGx 1919-8F (28) and TGx 1448-2E (30) in group 5; TGx 1903-5F (15) in group 6; TGx 1904-4F (11) and TGx 1910-14F (12) in group 7.

Fig.2 presents the molecular dendrogram, based on the similarity coefficients among the forty soybean accessions, as revealed by UPGMA cluster analysis based on the RAPD markers. The dendrogram shows a clear separation of the genotypes into five groups. For instance, TGx 1909-3F (1) and TGX 1910-8F (28) were in group 1, while TGx 1904-6F (2), UG-5 (10), TGx 1903-3F (36), TGx 1908-9F (19) were in group 2. The majority of the accessions that were evaluated were clustered in group 3, while groups 2 and 4 had five and six accessions, respectively. These groups (apart from groups 1 and 5) could be further divided into sub-groups at different similarity levels.

DISCUSSION

This study assessed the morphological and RAPD characterization of forty soybean accessions (*Glycine max* (L.) Merr. The analysis of morphological traits revealed that there is a possibility for selection among the accessions for the seven traits evaluated. In addition, there is a need to evaluate the soybean in two or more locations for seed yield to arrive at a logical conclusion with regards to the selection for this trait. According to Funnah and Mak (1980), no valid comparisons could be made regarding the relative performance of crop genotypes over all the environments in the presence of genotype by environment interaction.

The significant and positive associations observed between the seed yield per plot and the number of pods per plant, pod length and pod yield per plant indicated that these traits have influence on seed yield per plot and could be used as the selection criteria in soybean breeding programme. However, reduced days to maturity and days to 50% flowering tend to increase seed yield in soybean as it is negatively correlated to the number of pods per plant, pod length and 100-seed yield.

The result of the principal component analysis showed that different characters contributed differently to the total variation in the soybean genotypes, as indicated by the Eigen value as well as their weight and loading in different principal axes. The first principal component that accounted for the highest proportion of the total variation indicated the contribution of seed yield per plot, pod yield per plant, number of pods per plant and pod length to grain yield in soybean. If selection was to be made between the cluster groups for a future breeding exercise, these traits should then be given high priorities.

The cluster analysis had singular efficacy and ability to identify crop genotypes with the highest level of similarity through the dendrogram generated (Aliyu *et al*., 2000). The evaluation of phenetic diversity within soybean genotypes, using the cluster analysis in this study, provided seven clusters which were re-ordered into three clusters by the PCA analysis, with a lot of variations in the morphological properties. Mehetre *et al*. (1994) evaluated 51 soybean genotypes and found 10 clusters. Das *et al*. (2001) reported that the grouping pattern of the diverse genotypes suggested no parallelism between genetic divergence and geographical distribution of the genotypes. Ghatge and Kadu (1993) evaluated 58 soybean genotypes from diverse eco-geographical areas, and observed seven clusters on the basis of yield components. Meanwhile, Kumar and Nadarajan (1994) studied genetic diversity for yield components in 64 soybean genotypes, and revealed 11 clusters. Ihsan Ullah *et al*. (2007) reported 5 clusters derived from 10 genotypes on the basis of seed yield in sunflower.

Broschat (1979) considered PCA as a powerful technique for data reduction as it removes interrelationships among the components. The results reported by various researchers showed the multivariate analysis as a valid system to deal with germplasm collection. Rabbani *et al*. (1998) determined the extent of diversity and the relationship among

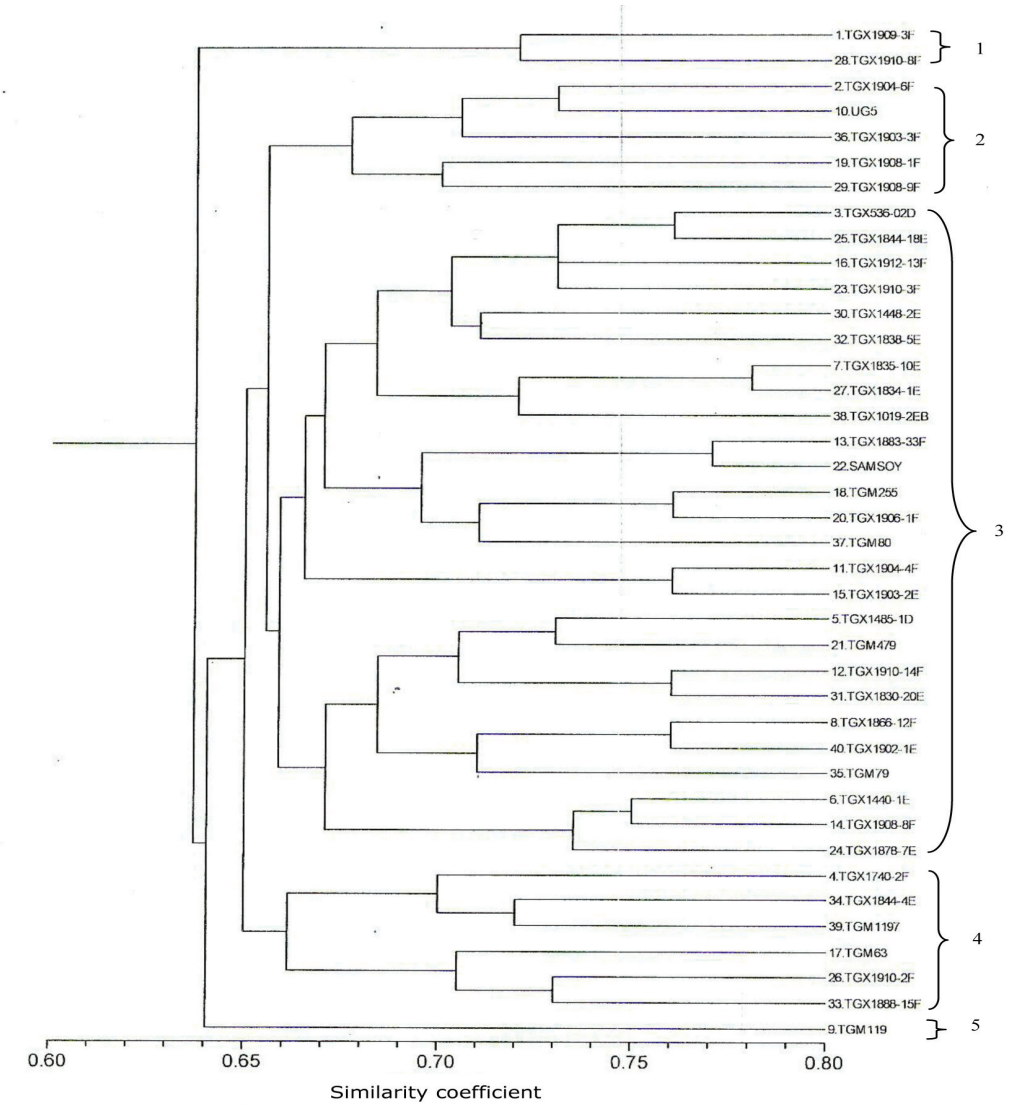

Fig. 2: Molecular dendrogram showing the genetic similarity among 40 soybean accessions revealed by UPGMA cluster analysis based on RAPD markers

Brassica juncea germplasm from Pakistan for 35 morphological characters in 52 accessions using the cluster and principal component analysis. Meanwhile, Ghafoor *et al*. (2001) studied genetic diversity in blackgram germplasm accessions. Quantitative traits were analysed for the cluster and principal component analysis. Iqbal *et al*. (2008) conducted cluster and principal component analyses on soybean germplasm. The five clusters observed on the dendrogram were reduced to only three when PC1 was plotted against PC2.

Although the morphological dendrogram generated from the similarity or distance matrices had provided an overall pattern of variation as well as the degree of relatedness among genotypes, the variations in environmental conditions such as soil types, and soil fertility

TABLE 5
Characteristics pattern of seven groups of soybean genotypes showing their mean values with standard deviations in parentheses

Character	I 14, 17, 20, 24, 25, 3, 32, 33, 36, 5	II 2, 34, 40, 6	III 13, 18, 19, 22, 28, 30, 7, 8	IV 4	V 15, 35	VI 23, 29, 38, 39, 9	VII 1, 10, 11, 12, 16, 21, 26, 27, 31, 37	Min	Max
Days to 50% flowering	42.10 (2.47)	43.84 (2.30)	41.52 (2.17)	42.00 (0.00)	42.75 (1.06)	42.75 (1.09)	42.52 (0.85)	41.52	43.84
Days to maturity	86.78 (2.63)	87.79 (3.26)	86.11 (2.22)	86.50 (0.00)	85.00 (2.12)	87.47 (0.77)	86.43 (0.98)	85.00	87.79
Number of pods per plant	18.97 (2.10)	13.63 (1.83)	17.38 (1.54)	12.42 (0.00)	19.96 (1.12)	14.33 (0.94)	15.44 (1.52)	12.42	19.96
Pod length	4.54 (0.23)	3.84 (0.28)	4.50 (0.27)	3.38 (0.00)	4.89 (0.09)	4.10 (0.28)	4.27 (0.31)	3.38	4.89
Pod yield per plant	5.88 (0.33)	3.56 (0.39)	5.15 (0.43)	3.52 (0.00)	6.57 (0.13)	4.11 (0.30)	4.69 (0.38)	3.52	6.57
100-seed weight	12.59 (1.10)	9.25 (1.69)	11.02 (0.96)	9.09 (0.00)	13.13 (1.87)	11.25 (2.17)	11.41 (2.54)	9.09	13.13
Seed yield per plot	429.87 (12.33)	252.04 (13.56)	375.13 (15.89)	211.02 (0.00)	468.58 (12.76)	295.98 (5.62)	339.51 (10.86)	211.02	468.58

TABLE 6
Nucleotide sequence of the selected primers with the number of amplified products and fragment size range (bp)

Primer	Sequence 5' to 3'	Polymorphic bands	Monomorphic bands	Fragment size range (bp)
OPG 11	TGCCCGTCGT	4	4	500 – 2000
OPI 15	TCATCCGAGG	6	5	250 – 2500
OPJ 05	CTCCATGGGG	7	3	500 – 1750
OPK 11	AATGCCCCAG	8	2	500 – 2000
OPL 13	ACCGCCTGCT	5	4	400 – 3000
OPP 12	AAGGGCGAGT	7	4	800 – 3500
OPS 18	CTGGCGAACT	8	2	300 – 2000
OPU 16	CTGCGCTGGA	10	2	500 – 2500
OPX 04	CCGCTACCGA	7	3	750 – 2500
OPY 20	AGCCGTGGAA	7	2	500 – 2000
Total		69	31	

levels (Steel, 1972), light, temperature and moisture regime (Sumerfield & Huxley, 1973; Morakinyo & Ajibade, 1998), allow for different results to be obtained using morphological grouping, particularly when the experiments were repeated in time and/or space. The observations above tend to emphasize the superiority and convincing discriminatory evidence of molecular grouping over and above the morphological grouping. The five major clusters of the RAPD dendrogram, together with their internal groups, demonstrated the polymorphic nature of the 40 soybean genotypes used in the current study.

The dendrogram obtained from the RAPD markers revealed that the markers were more discriminatory, highly polymorphic, and thus, more informative than the one obtained from the morphological characters because the markers were based on the ten OPERON primers to generate 69 RAPD bands across the 40 soybean accessions. Moreover, most of the bands were polymorphic and each band was able to differentiate between at least two of the 40 soybean accessions. Thus, differentiation among the soybean genotypes was higher using RAPD markers than the morphological characters. RAPD is a valuable tool for assessing genetic diversity levels in vegetable soybean. It detects polymorphism at the DNA level and thus is more efficient in discrimination among the varieties (Chowdhury *et al*., 2001).

In conclusion, both morphological and genetic variations were found to exist among the 40 soybean accessions that were evaluated. The number of pods per plant, pod yield per plant, 100-seed weight and pod yield per plot contributed a greater proportion of the variations that existed among the cluster groups. The dendrogram obtained from the molecular markers was more discriminatory than the one obtained from the morphological characters because the markers were based on 69 polymorphic markers produced by ten primers. The molecular dendrogram clustered the 40 soybean accessions into five groups, whereas the one based on the morphological characters had seven. The Principal Component Analysis indicated that all the 40 soybean accessions were ordered into three distinct PCA clusters.

The present study has indicated TGx 1834-1E and TGx 1910-2F as high yielding genotypes, and accessions TGM 80 and TGx 1830-20E as early maturing genotypes; however, it is unlikely that these accessions are the best to be found in the germplasm. Thus, collection, conservation and further evaluation for the selection of better germplasm are essential.

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Amphibian Biodiversity of Gunung Inas Forest Reserve, Kedah, Malaysia

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ABSTRACT

A study on the biodiversity of frogs and toads from Compartment 15 of Gunung Inas Forest Reserve, Kedah, was carried out for a 6 month period, beginning August 2008 till end of January 2009. Samplings were conducted once a month comprising a total catch effort of about 85 man-hours. Positive identification of specimens follows that of Berry (1975), Sukumaran (2006) and Norhayati *et al*., (2009). Twenty-eight species of anurans from six families were found to inhabit the site. The two most abundant species were *Amolops larutensis* and *Phrynoidis aspera*. Thirteen species namely *Megophrys nasuta*, *Leptobrachium hendricksoni Duttaphrynus melanostictus, Limnonectes malesianus, Limnonectes laticeps, Limnonectes plicatellus, Hylarana doriae, Hylarana erythraea*, *Hylarana luctuosa, Humerana miopus, Hoplobatrachus rugulosus, Rhacophorus tunkui,* and *Nyctixalus pictus* were considered rare. The Shannon-Wiener Diversity Index (H') was low at 0.745, while the Evenness Index (J) was low with the value of 0.149. The presence of clean water species, such as *Hylarana luctuosa, Hylarana labialis, Odorrana hosii*, and *Phrynoidis aspera* showed that the habitat at Compartment 15 of Gunung Inas Forest Reserve was largely undisturbed and pristine. It is hoped that future development of the site into a recreational facility does not degrade the uncontaminated riparian ecosystem that is essential for amphibian survival.

Keywords: Amphibians, biodiversity, primary forest, Gunung Inas, Malaysia

INTRODUCTION

The amphibian fauna of Peninsular Malaysia is rich in biodiversity, comprising 4 caecilians and about 88 frog species and toads from 5 families, namely Megophrydae, Bufonidae, Rhacoporidae, Ranidae and Microhylidae (Berry, 1975; Kiew, 1984). In Borneo, there are 138 species of amphibian which consist of anuran only (Inger & Tan, 1996). Research by Ramlah (2002) in Sedilu Mangrove Forest Reserve, Sarawak found 11 species of frogs. The research resulted in low diversity in Sedilu mangrove Forest Reserve (H=2.40). The most dominant species in the area were *Rana baramica, Limnonectes paramacrodon, Occidozyga laevis*, and *Bufo quadriporcatus*. Das (2006) found 59 anuran species from 5 families at the Crocker Range in Sabah and most of them are montane species. Another research conducted by Ibrahim *et al*.,(2006)in Gunung Jerai, Kedah yielded 85 individuals from 14 species in 4 families.

Populations of amphibians and reptiles are declining due to among others, habitat degradation, pollution, deforestation and diseases (Doherty-Bone, 2008) and Ibrahim *et al*., (2006) and Kiew (1984) reported that

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forest frog species are threatened by logging and development, and are thus, vulnerable to extinction. This is especially so since of late, a tremendous increase of deforestation has been noticed to occur in Peninsular Malaysia. Forest cover has fallen dramatically in Malaysia since the 1970s. While Food and Agriculture Organization (FAO) says that forests still cover more than 60% of the country, only 11.6% of these forests are considered pristine (Butler, 2005). Hence, it is vital that we undertake basic studies on amphibian numbers and populations before the force of progress and development push these creatures into oblivion. Other than inventories, checklists and surveys, very little information is available on the amphibian fauna in Malaysia, but we do need to do this kind of studies first before we can shift to other aspects of their ecology. Therefore, this paper reports on the diversity of amphibians found in Compartment 15 of Gunung Inas Forest Reserve as baseline data for future studies of amphibians in the general vicinity.

MATERIALS & METHOD

Study Site

Compartment 15 of Gunung Inas Forest Reserve is located at 5° 32' 60" N and 100° 35' 60" E with elevation 100m above sea level. It is situated 30 km due east of Kulim, Kedah. The forest coverage is about 36 979 ha. The study area is a lowland dipterocarp forest, which is a very suitable habitat for a diverse amphibian fauna to strive. A fast flowing, rocky river, Sungai Sedim, flows through the compartment arising from Gunung Inas and emptying into Sg. Muda. Among the dominant plant species here are *Shorea curtisii* (Dark Red Meranti), *Shorea leprosula* (Red Meranti), *Shorea macroptera* (Light Red Meranti), *Scorodocarpus borneensis* (Bawang Hutan), *Artocarpus lanceifolius* (Keledang) and *Callophyllumm* spp. (Bintangor). The dense vegetation on the forest floor and the presence of Sungai Sedim are ideal for certain species of frogs that breed in the streams. There are a lot of activities and

Fig.1: Location of Compartment 15, Gunung Inas Forest Reserve, Kedah, Malaysia, (5° 32' 60" N, 100° 35' 60" E). Source: Google maps (2010).

projects running now in Gunung Inas Forest Reserve such as Sedim Recreational Forest that comprises chalets, hostels and tourists attraction the Tree Top Walk. Among the activities there are picnics, camping, jungle trekking, water rafting and kayaking.

Sampling Method

Samplings were done during night time and specimens were caught by using net or by hand. Field parties comprising 5-6 persons scour along 200m of the riparian zone of Sungai Sedim and along trails in the forest from 2000h to 2200h. When captured, frogs were placed in individual plastic bags and transported to laboratory for processing where the morphometric data of individuals for every species was recorded. Frogs were identified by referring to Berry (1975), Sukumaran (2006) and Norhayati *et al.,* (2009). The data were analyzed by using various statistical methods including Shanon-Wiener Index for species diversity, Menhinick Index and Margalef Index for species richness, Evenness Index, and Simpson Index for species dominance (Khang, (2006)).

Man-hour calculation:
Number of individual x total of hours = Man-hours

RESULTS AND DISCUSSION

A total of 932 anuran specimens comprising 28 species from six families were collected during the study, (Table 2).

The two most abundant species were *Amolops larutensis* and *Phrynoidis aspera*. These two species were all collected along the riparian zone of Sungai Sedim River. The value for Margalef Index (M=10.44) and Menhinick Index (1.04 is significantly high and thus prove that the study area is rich with anuran species. The value for Shannon-Wiener Index is 0.75 and the value of H'max is 5 which shows that the area has a low diverse anuran community. This low H'value (low diversity) is due to the high number of individuals of the species *Amolops larutensis* and *Phrynoidis aspera* and they skewed the analysis of Shannon-Wiener index towards the low side The value for Evenness Index (J) is low at 0.15. The value of Simpson index is 0.31 which indicates low species dominance in Compartment 15. Thus from statistical analysis we can conclude that the amphibian assemblage at Compartment 15 Gunung Inas Forest Reserve has a high species richness, low species diversity, and low species evenness. The most abundant species are *Amolops larutensis* with 514 individuals

TABLE 1
Man-hours calculation.

No. of individual involved	Total number of hours	Total hours x man-power
6	1 hour	6 hours
4	1 hour 15 minutes	5 hours
6	1 hour 55 minutes	11 hours 30 minutes
5	2 hours 5 minutes	10 hours 25 minutes
5	2 hours 20 minutes	11 hours 40 minutes
4	1 hour 15 minutes	5 hours
1	1 hour	1 hour
2	1 hour 10 minute	2 hours 20 minutes
2	3 hours	6 hours
7	2 hours	14 hours
5	2 hours 25 minutes	12 hours 5 minutes
Total hours of sampling		85 man-hours

TABLE 2
List of species and number of individuals collected.

No	Family	Species	Common name	Total
1	Megophrydae	*Megophrys nasuta*	Malayan Horned Frog	1
		Leptobrachium hendricksoni	Spotted Litter Frog	33
2	Bufonidae	*Pedostibes hosii*	Yellow Spotted Tree Toad	5
		Ingerophrynus parvus	Dwarf Lesser Toad	20
		Duttaphrynus melanostictus	Common Sunda Toad	3
		Phrynoidis aspera	Giant River Toad	216
3	Dicroglossidae	*Fejervarya limnocharis*	Paddy Frog	13
		Fejervarya cancrivora	Crab-eating Frog	9
		Limnonectes plicatellus	Rhinoceros Frog	3
		Limnonectes blythii	Blyth's Giant Frog	9
		Limnonectes malesianus	Peat Swamp Frog	2
		Limnonectes laticeps	Flat-Headed Frog	1
		Occidozyga laevis	Yellow-bellied puddle Frog	17
		Occidozyga lima	Green Puddle Frog	8
4	Microhylidae	*Microhyla heymonsi*	Taiwan Rice Frog	9
5	Ranidae	*Hylarana erythraea*	Green Paddy Frog	8
		Hylarana luctuosa	Mahogany Frog	1
		Hylarana nicobariensis	Cricket Frog	4
		Hylarana labialis	White-lipped Frog	10
		Hylarana doriae	Doria's Frog	1
		Humerana miopus	Diagonal-lined Frog	2
		Hoplobatrachus rugulosus	Chinese Edible Frog	1
		Amolops larutensis	Larut Torrent Frog	514
		Odorrana hosii	Poison Rock Frog	35
		Odorrana monjerai	Mount Jerai Frog	15
6	Rhacophoridae	*Polypedates leucomystax*	Common Tree Frog	16
		Rhacophorus tunkui	Tunku's Tree Frog	8
		Nyctixalus pictus	Cinnamon Tree Frog	1
Total				932

TABLE 3
Habitat of amphibians found in Gunung Inas Forest Reserve

No	Species	Location (Habitat)	Sizes
1	*Megophrys nasuta*	Found on the forest floor or rocks where they blend in well with dead leaves	70-105mm (males); 90-125mm (females)
2	*Leptobrachium hendricksoni*	Found on the ground, on jungle trails and road cuts. (mostly under leaf-litters)	39-48mm (males); 52-80mm (females)
3	*Pedostibes hosii*	Found on tree branches and on rocks	53-80mm (males); 89-105mm (females)
4	*Ingerophrynus parvus*	Found along riverbanks of small stream to rivers and along the forest tracks.	Up to 52 mm
5	*Duttaphrynus melanostictus*	Found mostly at disturbed area and human settlements.	57-83mm (males), 65-85mm (females)
6	*Phrynoidis aspera*	Found along river trails and also in jungle trails.	70-100mm (males), 95-140mm (females)
7	*Fejervarya limnocharis*	Found in disturbed area (example: villages, paddy field and garden)	32-50mm (males); 48-60mm (females)
8	*Fejervarya cancrivora*	Found along the coast, lower reaches of large river basins, semi brackish, swampy areas close to the sea or in freshwater swamps beyond tidal influence.	51-70mm (males); 53-82mm (females)
9	*Limnonectes plicatellus*	Found in lowland swamp forest areas with small rivers and streams.	35-43mm (males); 29-34mm (females)
10	*Limnonectes blythii*	Found along rivers and streams in lowland forests but can also be found in disturbed areas and hill forests.	90-260mm (males); 85-125mm (females)
11	*Limnonectes malesianus*	Found along jungle trails and disturbed forests.	70-150mm (males); 75-95mm (females)
12	*Limnonectes laticeps*	Found along river trails and small streams.	24-47mm (males); 32-46mm (females)
13	*Occidozyga laevis*	Found in shallow pools and puddles in lowland forests and slightly disturbed areas.	(26-62mm)
14	*Occidozyga lima*	Found in shallow pools and puddles in lowland forests and slightly disturbed areas.	(20-25mm)
15	*Microhyla heymonsi*	Found in cleared areas and disturbed forests on low bushes on the ground among grasses, shrubs and other vegetation.	16-21mm (males); 22-26mm (females)
16	*Hylarana erythraea*	Found in rice fields, disturbed areas and swampy areas.	30-45mm (males); 50-75mm (females)
17	*Hylarana luctuosa*	Found on rocks, steep banks or low vegetation very near flowing waters of clear upland rivers.	41-59mm (males); 42-60mm (females)
18	*Hylarana nicobariensis*	Found in swampy areas of lowland secondary forests or at disturbed areas.	37-47mm (males); 47-53mm (females)
19	*Hylarana labialis*	Found in forest streams and swamps.	37-48mm (males); 44-71mm (females)

Table 3 (*continued*)

20	*Hylarana doriae*	Found moist lowland forests, subtropical or tropical moist montane forests, and rivers.	70-150mm (males); 75-95mm (females)
21	*Humerana miopus*	Found at forest floor in swampy areas.	(71-73mm)
22	*Hoplobatrachus rugulosus*	Found in swampy areas and paddy fields.	(84-77mm)
23	*Amolops larutensis*	Found along forest streams and rivers and usually on rocks.	35-45mm (males); 53-75mm (females)
24	*Odorrana hosii*	Found on rocks, steep banks or low vegetation near flowing waters.	45-63mm (males); 85-100mm (females)
25	*Odorrana monjerai*	Found along clear, moderate to swift flowing forest streams and waterfalls in lowland or upperhill forests.	75mm (females); 38-43mm (males)
26	*Polypedates leucomystax*	Found near human settlements, disturbed areas, and near water bodies (if breeding).	37-50mm (males); 57-75mm (females)
27	*Rhacophorus tunkui*	Found in lowland forests, on leaves or branches surrounding puddles and forest ponds.	up to 42 mm
28	*Nyctixalus pictus*	Found in primary and secondary forest as well as slightly disturbed habitats, on leaves and branches of small trees low to the ground	Up to 33 mm (males); up to 38mm (females)

TABLE 4
Comparison of amphibian diversity in Northern Malaysia.

No. of Species	Locality	Source
26	Island of Penang	Ibrahim *et al*. (2008)
19	South Kedah	Shahriza (2007)
16	Island of Langkawi, Kedah	Ibrahim *et al*. (2006)
24	Island of Langkawi, Kedah	Grismer *et al*. (2006)
56	Ulu Muda Forest Reserve, Kedah	Norhayati *et al* (2005)
36	Ulu Muda Forest Reserve, Kedah	Norsham *et al*. (2005)
13	Wang Kelian State Park, Perlis	Ibrahim *et al.* (2001)
9	Belum Forest Reserve, Perak	Norsham *et al.* (2000)
24	Temenggor Forest Reserve, Perak	Kiew *et al*. (1995)
28	Gunung Inas Forest Reserve	This Study

TABLE 5
Relative abundance of amphibians in Compartment 15 of Gunung Inas Forest Reserve

No	Species	Common Name	Relative Abundance (%)
1	*Megophrys nasuta*	Malayan Horned Frog	0.1
2	*Leptobrachium hendricksoni*	Spotted Litter Frog	3.5
3	*Pedostibes hosii*	Yellow Spotted Tree Toad	0.5
4	*Ingerophrynus parvus*	Dwarf Lesser Toad	2.2
5	*Duttaphrynus melanostictus*	Common Sunda Toad	0.3
6	*Phrynoidis aspera*	Giant River Toad	22.5
7	*Fejervarya limnocharis*	Paddy Frog	1.4
8	*Fejervarya cancrivora*	Crab-eating Frog	1.0
9	*Limnonectes plicatellus*	Rhinoceros Frog	0.3
10	*Limnonectes blythii*	Blyth's Giant Frog	1.0
11	*Limnonectes malesianus*	Peat Swamp Frog	0.2
12	*Limnonectes laticeps*	Flat-Headed Frog	0.1
13	*Occidozyga laevis*	Yellow-bellied puddle Frog	1.8
14	*Occidozyga lima*	Green Puddle Frog	0.9
15	*Microhyla heymonsi*	Taiwan Rice Frog	1.0
16	*Hylarana erythraea*	Green Paddy Frog	0.9
17	*Hylarana luctuosa*	Mahogany Frog	0.1
18	*Hylarana nicobariensis*	Cricket Frog	0.4
19	*Hylarana labialis*	White-lipped Frog	1.1
20	*Hylarana doriae*	Doria's Frog	0.1
21	*Humerana miopus*	Diagonal-lined Frog	0.2
22	*Hoplobatrachus rugulosus*	Chinese Edible Frog	0.1
23	*Amolops larutensis*	Larut Torrent Frog	55.2
24	*Odorrana hosii*	Poison Rock Frog	3.8
25	*Odorrana monjerai*	Mount Jerai Frog	1.6
26	*Polypedates leucomystax*	Common Tree Frog	1.7
27	*Rhacophorus tunkui*	Tunku's Tree Frog	0.9
28	*Nyctixalus pictus*	Cinnamon Tree Frog	0.1
		Total	100

followed by *Phrynoidis aspera* with 216 individuals. The presence of clean water species such as *Hylarana luctuosa, Hylarana labialis* and *Odorrana hosii* shows that the habitat at compartment 15 of Gunung Inas Forest Reserve is largely undisturbed and pristine. Even as we were conducting the study, the state government, in cooperation with the Ministry of Tourism, Malaysia, is developing the area into a large recreational facility by building chalets, roads and parking areas in the vicinity. The beautiful Cinnamon Tree Frog (*Nyctixalus pictus*) which is classified as near threatened under the IUCN 'Red List' status is also found here, as well as 3 other near threatened species, namely *Limnonectes Blythii*, *Limnonectes malesianus* and *Rhacoporus tunkui*. It is imperative that Gunung Inas Forest Reserve be preserved in its pristine state since it harbours a good number of amphibian species. This forest reserve and others adjacent to it such as Ulu Muda Forest Reserve to the north and Bintang Hijau Forest Reserve to the south are rich in amphibian species (Norhayati *et al*., 2005). It is hoped that the future development of the site into a recreational facility does not compromise or degrade the uncontaminated ecosystem that is essential for amphibian survival and existence since they are known to play important roles in the ecological processes of the tropical forest ecosystem.

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Molecular Evidence in Identifying Parents of *Garcinia mangostana* L.

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ABSTRACT

The obligate apomicts *Garcinia mangostana* L. (Clusiaceae) was described from Malaysia and hypothesized to have originated from the natural hybridization between *G. malaccensis* and *G. hombroniana.* One of the parents, i.e. *G. malaccensis,* was believed to be endemic in the state of Melaka. However, this was determined only through a comparison of their chromosome number and morphological characteristics. There is still the possibility of other species within the same section of *G. mangotana* as the possible parents. Thus, investigations were carried out using molecular markers from three different regions of the internal spacer, chloroplast and micorsatellite. The objective of this study was to identify the possible parents of *G. mangostana* by comparing its relationship to other species within the same section, based on the genetic analysis of the internal spacer, chloroplast and microsatellite regions. Meanwhile, comparisons of allele sizes between *G. mangostana* with *G. malaccensis, G. opaca* and *G. hombroniana* using six polymorphic primers which had previously been developed were also performed. For phylogenetic analysis ITS, trnL and accD-psaL primers were used to determine the relationships between the four *Garcinia* species in the *Garcinia* section with two other sections and the genus *Clusia* as an outgroup. From the genetic analysis, it was found that *G. hombroniana* shares no common allele with the other species, while *G. opaca* has similar allele sizes with *G. mangostana* and *G. malaccensis*. The phylogenetic tree also showed that the closest relative to *G. mangostana* is *G. opaca* and *G. malaccensis*. This proves that *G. opaca* is more likely to be the other parent of *G. mangostana* rather than *G. hombroniana*.

Keywords: ***Chloroplast, phylogenetic, mangosteen, microsatellite***

INTRODUCTION

The origin of the apomictic and totally female *Garcinia mangostana* has been suggested to be from Malaysia. Based on the records by Whitmore (1973), one of the determined parents, i.e. *Garcinia malaccensis*, was only found in the state of Melaka. *G. mangostana* might have arisen sexually from the hybridization of this particular species with any other species that is from the same taxonomic section, i.e. Section *Garcinia*. According to Richards (1990b), *G. mangostana* might be a hybrid between *G. hombroniana* and *G. malaccensis*. The former species is sometimes cultivated, but *G. malaccensis* is only known as a wild plant. Richards (1990b) believed that only a single apomictic female arose after a hybridization event between the facultative apomicts, and that

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no sexual reproduction subsequently came after that. Consequently, no males have been reported in this species, apart from the single male which was last reported by Idris and Rukayah (1987) in Peninsular Malaysia. Although the tree and flower which were reported by Idris and Rukayah (1987) possess similar morphological characteristics to female *G. mangostana*, the flower has numerous stamens with pollen grains. Nonetheless, it was uncertain that the male tree was a hybrid of *G. hombroniana* with *G. malaccensis*. The possibility of *G. mangostana* back-crossing to either one of the parents should therefore be considered. However, further studies were not possible as the tree is no longer available. Individuals in the genus *Garcinia* are able to reproduce via apomixis, regardless of whether they are facultatively or obligately apomictic. Without the existing hybrids having been recorded, and having the means to reproduce through obligate apomixis, all *G. mangostana* are considered as the components of a clone containing no genetic variation within or between populations. This generates a negative impact towards *G. mangostana* as one of the economically important fruit as it lacks a number of varieties or cultivars as compared to other commercial fruit such as durian and rambutan. Another drawback that made *G. mangostana* the least planted fruit tree among the local growers is that they have a long juvenile period which usually takes ten years for a *G. mangostana* tree to mature and to bear fruit. By identifying the parents of *G. mangostana*, hybridization could be carried out once again to produce and screen for progeny with favourable traits, especially one with a shorter juvenile period. As suggested by Richards (1990b), sexual reproduction might have occurred between the diploid *G. hombroniana* and diploid *G. malaccensis* to produce the tetraploid *G. mangostana.* This was concluded from chromosome number, where *G. hombroniana* was n=24 (2n=48), as reported by Richards (1990a), and the chromosome count for *G. malaccensis* reported by Ha (1988) was 2n=42-43. The chromosome counts for *G. mangostana* have been speculative, as there is only one study, i.e. by Ha (1988), which has reported the chromosome count of *G. mangostana* as 2n=?90. This would be logical if the parents were *G. hombroniana* and *G. malaccensis.* Richards (1990b) also compared the morphological characteristics of the three species which gave strong support to the theory that *G. hombroniana* and *G. malaccensis* are the parents. However, the researcher did not compare the chromosome counts to other possible species within the same Garcinia section which could lead to the possibility of other species as being the possible parents. These include *G. penangiana* and *G. opaca* which have strong phenotypic resemblance to *G. hombroniana*. Phylogenetic studies, based on the Internal Transcribed Spacer (ITS) sequences of the nuclear ribosomal regions carried out by Nazre (2007), showed that *G. mangostana* is closely related to *G. hombroniana, G. malaccensis* and *G. opaca*, indicating that all three species have the possibility of being one of the parents. The methods for parentage analysis include exclusion, categorical and fractional likelihood, and genotyping reconstruction (Jones & Ardren, 2003). Although these methods may be an ideal and convincing way to determine the parents of *G. mangostana*, screening for the incompatibilities between the parents and offspring will consume a long period of time, especially for a long juvenile period species like *Garcinia* spp. Phylogenetic analysis which has always been the method of choice for determining the relationships among the species. Two DNA regions are commonly associated with phylogenetics, i.e. the internal transcribed spacer (ITS) and chloroplast regions. The nucleotide sequence variations found in each of the ITS sequences were often best suited for comparing species and closely related genera (Saar & Polans, 2000; Soltis & Soltis, 1998). The internal transcribed spacer region and the intergenic spacer of the nuclear rRNA repeat units evolve the fastest and may vary among the species within a genus or among the populations (White *et al*., 1990) and are more suitable for comparison of closely related taxa (Baldwin, 1995). The chloroplast (cp) genome has been shown to be maternally inherited in the majority

of angiosperms (Ennos *et al*., 1999; Palmer *et al*., 1988). According to Haruki *et al*. (1998), cpDNA markers have been successfully used for tracing the maternal parent in *Lilium* species. However, the cladistic analyses may not be able to completely resolve reticulate relationships and may not be useful for identifying hybrids or parental species (McCade, 1992). For most biological systems, the most powerful genetic tools for parentage analysis are the microsatellite markers (Jones & Ardren, 2003). The paternity of parentage analysis can be achieved by any type of genetic markers provided that it is sufficiently polymorphic, and for that reason, microsatellites are usually preferred (Gerber *et al*., 2000). Microsatellite markers proved to be a powerful tool to study the relationship of species, cultivars or varieties as they are inherited in a co-dominant Mendelian manner. The study was carried out to identify the possible parents of *G. mangostana* by comparing its relationship to other species within the same section based on the genetic analysis of the internal spacer, chloroplast and microsatellite regions.

MATERIALS AND METHODS

Four species from the section Garcinia, namely *G. mangostana, G. hombroniana, G. opaca* and *G. malaccensis*, were compared in the DNA fragment analysis and 11 other species were included for the phylogenetic analysis (Table 1). DNA was extracted from the leaf samples of 15 *Garcinia* species using Qiagen Plant Mini Kit and quantified by comparing it to two DNA ladders of 20µg and 40µg on 1.2% agarose gel. Microsatellite loci were isolated from *G.*

TABLE 1
A list of *Garcinia* species examined and their sampling locations

No.	Botanical name	Taxonomic Section	Location of the samples
1	*G. atroviridis*	Brindonia	FRIM
2	*G. bancana*	Brindonia	Pasoh Forest Reserve
3	*G. cowa*	Brindonia	Pasoh Forest Reserve
4	*G. dulcis*	Xanthochymus	Pasoh Forest Reserve
5	*G. grifithii*	Brindonia	Pasoh Forest Reserve
6	*G. hombroniana*	Garcinia	Rimba Ilmu, UM
			FRIM, Kepong
			Pangkor Island
7	*G. malaccensis*	Garcinia	Pasoh Forest Reserve
			Sg. Menyala Forest Reserve
8	*G. mangostana*	Garcinia	UPM-Puchong Mangosteen Orchard
			UPM- Farm 10
			Raub
			Miri
			Melaka
9	*G. nervosa*	Xanthochymus	Pasoh Forest Reserve
10	*G. nigrolineata*	Brindonia	Pasoh Forest Reserve
11	*G. opaca*	Garcinia	Taman Negara Forest Reserve
12	*G. opacaE*	Garcinia	Pasoh Forest Reserve
13	*G. opacaR*	Garcinia	Pasoh Forest Reserve
14	*G. parvifolia*	Brindonia	Pasoh Forest Reserve
15	*G. prainiana*	Xanthochymus	UPM Puchong Mangosteen Orchard
16	*G. pyrifera*	Xanthochymus	Pasoh Forest Reserve
17	*G. sp1*	Unknown	Pasoh Forest Reserve

mangostana and seven micosatellite primer pairs were developed following the hybridization and enrichment techniques (Edwards *et al*., 1996) to produce six polymorphic primer pairs which were later used for the fragment analysis (Table 2). The DNA fragments were amplified in 10µl reaction mix; 1X *Taq* Buffer (16mM $(NH_4)_2SO_4$, 67 mM Tris-HCL, 0.01% Tween-20), 2.0mM $MgCl_2$, 0.2mM dNTP each, 0.2µM of each of the microsatellite primer pairs, 0.33µM (F) dCTP, 0.5U *Taq* (Bioline) and 0.5µl template DNA. The primers were labelled with (F)dCTP dyes and the dyes used were R6G(dCTP) and R110(dCTP), visualised as 'green' and 'blue' peaks respectively on the ABI 310. The PCR was performed in a thermal cycler (PTC-100, MJ Research, Inc.) and it consisted of an initial denaturation of 12min at 95°C, followed by 20 cycles for 15s at 95°C, 15s at annealing temperature, and 15s at 72°C, 10 cycles for 15s at 89°C, 15s at annealing temperature, 15s at 72°C, with a final elongation of 30min at 72°C. Purification of the PCR products was carried out by mixing the PCR product with 1µl NaAc 3M (pH4.6) and 22µl 99% ethanol and precipitated at -20°C for 5min. Finally, 1µl of the fluorescently labelled reaction products were combined with 12µl deionised formamide and 0.5µl GeneScan ROX500 size standard. The mixture was heated three minutes at 95°C and transferred into ABI tubes, and fragment analysis was carried out on an ABI PRISM® 310 Genetic Analyzer. For the ITS and chloroplast DNA analysis, amplifications were carried out in 50µl reaction mixtures containing 1X PCR buffer, 2mM $MgCl_2$, 10µM dNTPs, 0.2pmol/µl of each primer (Table 2), 1U taq polymerase and 5µl template DNA. PCR cycles were programmed on a PTC-100 DNA Engine (MJ Research, Inc.) following the protocol for each primer. The protocol for amplification with ITS/trnL primers follows the protocol for ITS: 1 cycle

TABLE 2
A list of the primers and their sequences

Primer	Sequence (5' → 3')	Source
GM1	F: GAGCAATCCCAATGGCTAAA	*
	R: CCGAGCTAAATGAATTGTGGA	*
GM2	F: TATGGAGCCTTTCGAGCCTA	*
	R: CACCTCAGATTTAGGCCATCA	*
GM5	F: TGATGAGAAACATGCAGTTGA	*
	R: TGATTCTGCAGCAT GGAAC	*
GM8	F: GTTTTGTCCCGGTTAAGTT	*
	R: AAGGGTTTGCAATGAACAG	*
GM10	F: GGCAACTGCTCCAAGTTAG	*
	R: TTTATCGGCCAAGTTATCG	*
GM11	F: TTGTGCTCTCTTCGCTCTT	*
	R: ATGGCAGTTTATTGCTTGG	*
ITS4	TCCTCCGCTTATTGATATGC	White *et al*. (1990)
ITS5	GGAAGTAGAAGTCGTAACAAGG	White *et al*. (1990)
trnL-c	CGAAATCGGTAGACGCTACG	Taberlet *et al*. (1991)
trnL-d	GGGGATAGAGGGACTTGAAC	Taberlet *et al*. (1991)
accD-769F	GGAAGT TTGAGCTTTATGCAAATGG	Small *et al*. (1998)
psaL-75R	AGAAGCCATTGCAATTGCCGGAAA	Small *et al*. (1998)

[*] *Primers developed from G. mangostana DNA region following Edwards et al. (1996) hybridization and enrichment techniques*

of initial denaturing at 94ºC for 3min, 30 cycles of denaturing at 94ºC for 10s, annealing at 55ºC for 20s, elongation at 72ºC for 1min and 30s and final elongation of 72ºC for 4min. Amplification using the accD-psaL primer follows the same protocol with minor modification of 1 cycle of initial denaturing at 94ºC for 5min, 30 cycles of denaturing at 94ºC for 30s, annealing at 50ºC for 30s, elongation at 72ºC for 2min and final elongation of 72ºC for 4min. Successful PCR amplification which produced a single DNA band was checked on 1.4% agarose gel. The amplified DNA was purified using the QIAGEN PCR purification kit and the DNA concentration of the purified PCR product was estimated on the gel by comparing it to a ladder of known concentration. Sequence reactions were carried out in both directions for each purified double-stranded PCR product using the Applied Biosystems Big Dye Terminator Ver. 1.1, buffer and primers. The sequence analysis was done on the ABI Prism 310 and the sequences were viewed and edited on Chromas Lite 2.0. The sequences were aligned by using ClustalX (vers. 1.8) and edited using ProSequence (Filatov 2002). The phylogenetic relationship analyses were conducted with PAUP, Version 4.0b (Swofford, 1999). The most parsimonious tree was obtained using the heuristic search option involving 100 replications of random addition sequence and tree bisection-reconnection (TBR) branch swapping. All characters were specified as equally weighted. The analyses were based on nucleotide substitutions; therefore, all gaps in the sequence were treated as missing data. Strict consensus and bootstrap analyses were conducted to assess the reliability of the tree. For bootstrap, 1000 replications were calculated using the heuristic search option with TBR branch swapping. Four to six closely related species of *Clusia sp.* were chosen as the outgroups (Table 3).

RESULTS AND DISCUSSION

The parent of *G. mangostana* was determined by comparing the allele size for six microsatellite loci of three *Garcinia* species Garcinia species with support analysis using the ITS and chloroplast markers. *G. mangostana* DNA samples from five different locations were compared and the results showed that all five samples had the same allele size for each locus. This further confirmed that the *G. mangostana* plants are genetically similar and are possible clones. Since *G. mangostana* are obligate apomicts and without the existence of male tree for possible pollination and fertilization, all *G. mangostana* are believed to be carrying the same genetic constituents, and hence, having the exact allele size for each locus. Meanwhile, facultative apomicts, such as *G. hombroniana* and *G. opaca*, are able to propagate via apomixis and also through sexual reproduction. Thus, genetic variations among accessions and populations exist. In this study, three samples of *G. hombroniana* from three different locations showed differences in the allele sizes for three loci, i.e. GM8, GM10 and GM11 (Table 4). This was also observed in *G. malaccensis* as there were differences in the allele size from their samples taken from two different locations. This finding proved that there are some degree of genetic variations among the accessions of *G. hombroniana*, *G. malaccensis* and *G. opaca*. Five loci were heterozygous and one locus (GM2) was homozygous. Two of the primers were able to amplify four alleles per locus in tetraploid *G. mangostana*, namely GM8 and GM11. Table 4 shows that *G. mangostana* shares three similar sized alleles with *G. malaccensis* at locus GM1, GM2 and GM11, with the allele sizes of 235, 237bp, 210, 211bp, and 171,173 bp, respectively. When compared to *G. opaca*, *G. mangsotana* was found to share allele sizes at two loci, namely, GM5 (101bp) and GM11 (171, 173, 193,195bp). Sharing of similar allele sizes was also observed between *G. malaccensis* and *G. opaca* at loci GM10 (133,149bp) and GM11 (171,173bp). Meanwhile, sharing of alleles between *G. mangostana* and *G. malaccensis*, between *G. mangostana* and *G. opaca*, as well as between *G. malaccensis* with *G. opaca* directly linked the three species in a closely unique relationship. The similarity of allele sizes between *G. mangostana* and *G.*

TABLE 3
A list of the *Clusia* species used as the outgroup for phylogenetics according to DNA region extracted from Genbank

Region	Clusia Species	Source	GenBank Accession number
ITS	*C. rosea*	Gehrig *et al*. (2003)	AJ509230
	C. minor	Gehrig *et al*. (2003)	AJ509208
	C. major	Gustafsson *et al.* (2002)	AY145222
	C. ducu	Gustafsson *et al.* (2002)	AY145220
	C. lanceolata	Gustafsson *et al.* (2002)	AY145195
	C. multiflora	Vaasen *et al*. (2002)	AJ414719
trnL-trnF intergenic spacer region	*C. rosea*	Hale *et al*. (2004)	AY144094
	C. multiflora	Hale *et al*. (2004)	AY144091
	C. lanceolata	Hale *et al*. (2004)	AY144085
	C. minorA	Hale *et al*. (2004)	AY144087
	C. ducuA	Hale *et al*. (2004)	AY144076
accD and psaL genes	*C. rosea*	Hale *et al*. (2004)	AY144017
	C. multiflora	Hale *et al*. (2004)	AY144013
	C. lanceolata	Hale *et al*. (2004)	AY144007
	C. major	Hale *et al*. (2004)	AY144008

malaccensis supports the idea that *G. malaccensis* is one of the parents of *G. mangostana*, as suspected by Richards (1990b). Interestingly, *G. hombroniana* shared no allele size with any of the three species. Instead, all the possible loci strongly suggested that *G. hombroniana* was not involved in contributing allele to *G. mangostana*. Although none of the allele sizes of *G. malaccensis* from the Pasoh population was similar to *G. mangostana*, this does not exclude *G. malaccensis* as one of the possible parents. The fact that *G. malaccensis* does not have the same reproductive system as *G. mangostana* and is facultatively apomicts, the occurrence of sexual reproduction within the population causes them to become more genetically diverse than *G. mangostana,* and thus, some individuals may not share the same alleles as *G. mangostana*. It seems that obtaining the right sample which matches the progeny is crucial. Therefore, it is essential to have a large sample size and to obtain the sex ratio of a population before sampling to recognize the pattern of apomixis or sexual reproduction in that population. To further support the idea that *G. malaccensis* and *G. opaca* are the possible parents for *G. mangostana*, phylogenetics analysis was carried out on both nuclear and chloroplast regions. Both the regions compare the mutation rate of nucleotides for *G. mangostana* in relation to *G. hombroniana*, *G. malaccensis* and *G. opaca*. Incorporating other species from various sectionsis generally required in phylogenetics to ensure accuracy of the phylogentic tree as well as to observe the relationships among the species from different sections. In this case, the genus *Clusia* was used as it was linked with close resemblance to *Garcinia*. The ITS sequences of *Garcinia* ranged from 640bp to 683bp, with an aligned length of 860bp. The phylogenetic analysis resulted in 1578 most-parsimonious trees of tree length (L) = 609, consistency index (CI) = 0.6470 ($CI_{uninformative}$ = 0.5928), retention index (RI) = 0.8184 and rescaled consistency index (RC) = 0.5295. Of 666 characters, a total of 225 (33.8%) characters were phylogenetically informative,

TABLE 4
Fragment analysis using six polymorphic primer pairs showing the allele sizes (bp) of four *Garcinia* species

Species	Population	Allele size at 5 loci					
		GM1	GM2	GM5	GM8	GM10	GM11
G. mangostana	UPM-Farm 10	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
	UPM-Puchong	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
	Raub	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
	Miri	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
	Melaka	235,237	210,211	101	233, 235, 242, 244	157,160	171,173, 193, 195
G. hombroniana	FRIM	n/a	217,219	n/a	209,209	313	183,183
	UM	n/a	217,219	n/a	207,209	314	181,183
	Pangkor	n/a	217,219	208	207,209	313	183,183
G. malaccensis	Pasoh	n/a	204	107,111	221	133,149	171,173
	Sg. Menyala	235,237	210,211	n/a	231	162	171,173, 193, 195
G. opaca	Rompin	n/a	n/a	149	n/a	133,149	n/a
	Pasoh	213	212,214	101	221	133,149	171,173, 193, 195

n/a – no amplification

71 variable characters are parsimony-uninformative and 370 (55.5%) characters were constant. The phylogenetic tree shown in *Fig. 1* indicates that all the *Garcinia* species were clustered accordingly to their respective section and the genus *Clusia* was the outgroup. The ITS data support three clades within *Garcinia*, with *G. bancana* not included in any one of them. Clade 1 (C1) contains the species from the section *Xanthochymus*, in clade 2 (C2) are the species from the section *Garcinia*, and clade 3 (C3) contains the species from section *Brindonia*. During leaf sampling in the Pasoh Forest Reserve, one tree was recorded as *G. eugeniaefolia* and eight trees were as *G. rostrata*. Both these species are from the section *Discostigma* (Jones, 1980). From the observations of leaf and flower morphologies, it was suggested that "*G. eugeniaefolia*" and "*G. rostrata*" are both of the same species with *G. opaca* (Nazre, pers.com). The BLAST inquiry from Genbank on the sequences of our collection, labelled as "*G. eugeniaefolia*" and "*G. rostrata*", was performed and shown as similar to *G. opaca* var *minor* with the E value of zero and the scores of 1106. The phylogram based on ITS sequences showed that "*G. eugeniaefolia*" designated as *G. opaca*E and "*G. rostrata*" designated as *G. opaca*R were in the same clade as *G. opaca* which is in section *Garcinia*. The results from the BLAST inquiry for *G. sp*1 showed a high similarity to *G. bancana*. Based on the phylogram illustrated in *Fig. 1*, *G. sp*1 is in the same clade as the species from section *Brindonia*, suggesting that G. *sp*1 is most probably *G. bancana.* Section *Garcinia*, which includes *G. mangostana*, *G. malaccensis*, *G. opaca* and *G. hombroniana*, showed that the number of changes in the branch length between *G. mangostana* with *G. malaccensis, G. opaca, G. opacaE, G. opacaR* and *G. hombroniana* was 19,17,9,5 and 21, respectively. This suggests that the closest species to *G. mangostana* are *G. opaca* and *G. malaccensis*. This also suggests that *G. opaca* and *G. malaccensis* are more likely to be the parents of *G. mangostana* than *G. hombroniana*, as suggested by Richards (1990b). The aligned trnL sequences were 707bp in length with the sequences varying from 640bp to 683bp. The phylogenetic analysis resulted in 1083 most-parsimonious trees of tree length (L) = 547, consistency index (CI) = 0.6216 ($CI_{uninformative}$ = 0.5651), retention index (RI) = 0.7214 and rescaled consistency index (RC) = 0.4484. One of the parsimonious trees is shown in *Fig. 2*. Of 671 characters, a total of 204 (30.4%) characters are phylogenetically informative, 62 (9.2%) variable characters are parsimony-uninformative and 405 (60.3%) characters are constant. Three major clades, which were recognized (C1-C4) with *G. atroviridis* and *G. bancana*, were not included in their expected group (*Fig. 2*). Clade 1 was section *Brindonia*, clade 2 section *Xanthochymus* and clade 3 section *Garcinia*. All the species in each clade otherwise agrees with Jones' (1980) classifications. The chloroplast data, like the previous ITS analyses, support that *G. opaca* and *G. malaccensis* is the closest sister to *G. mangostana.* The trnL chloroplast region of *Garcinia* contains mononucleotide repeats of the A nucleotide. The length of repeats ranged from 10 - 16 repeats at 113 – 132bp long. However, primers trnL-C and trnL-D were unable to amplify the DNA sample of *G. opaca*. It is important to note that the sequences used for this analysis might have been unreliable. This is because of the long A nucleotide repeats and the alignments of the sequences containing large gaps, notably from 387-480bp. Therefore, the phlygenetics analysis relies heavily on the other chloroplast region, i.e. the accD-psaL region. The length of accD-psaL DNA sequence ranged from 698bp to 753bp, with an aligned length of 798bp. The heuristic search yielded 401 trees length (L) =240, consistency index (CI) = 0.6125, homoplasy index (HI) = 0.3875, ($CI_{uniformative}$ = 0.4716), rescaled consistency index (RC) = 0.3882 and retention index (RI) = 0.6339. The phylogram can be divided into three clades (C1, C2 and C3) as shown in *Fig. 3*, and from the bootstrap analysis (*Fig. 4*), the monophyly of the ingroup was 100% supported. Of 681 characters, a total of 70 (10.3%) characters are phylogenetically informative, 61 (8.9%) variable characters are parsimony-unifnformative and 550 characters are constant. Clade 1 (76%

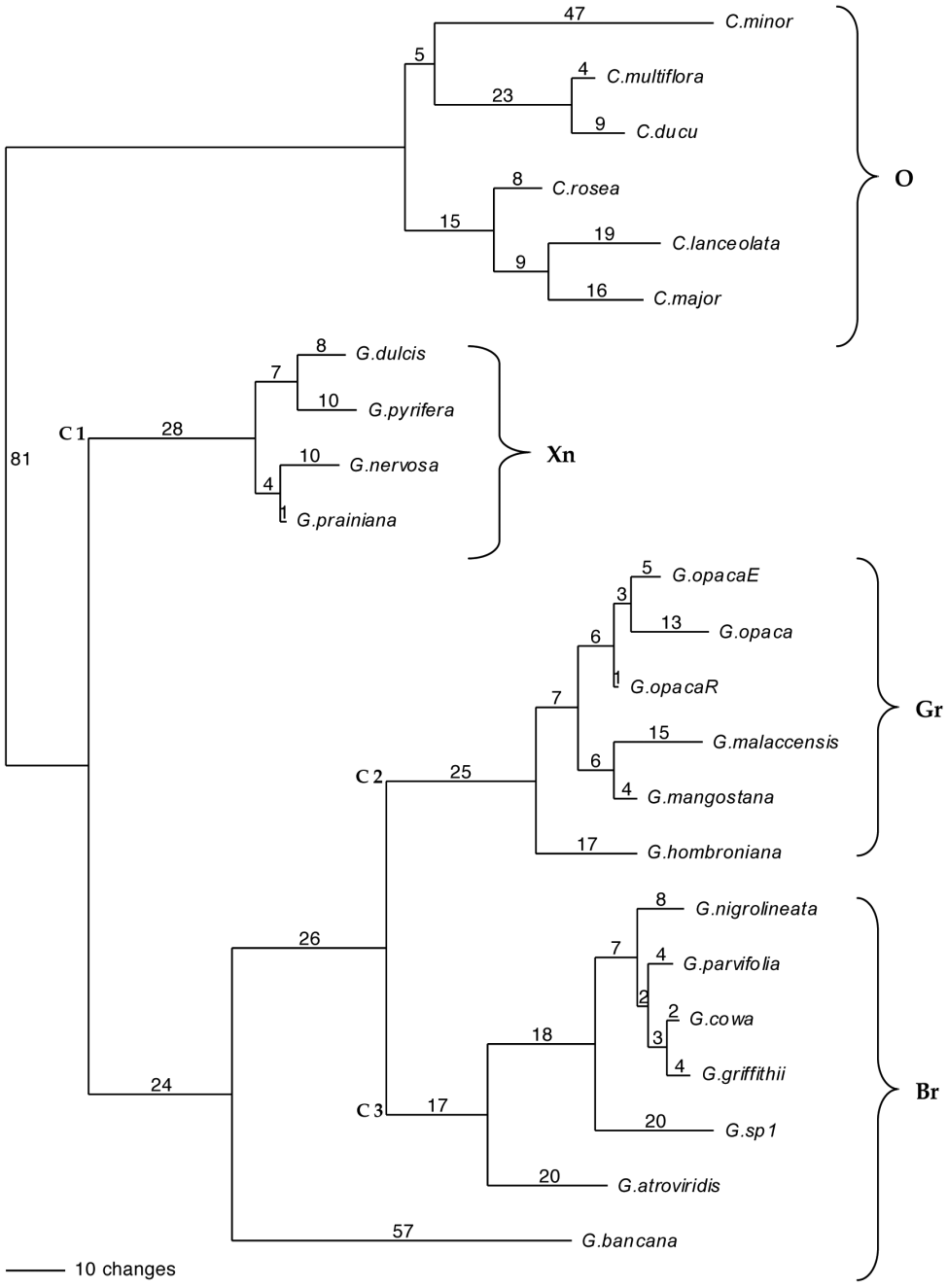

Fig. 1: One of most parsimonious trees based on the ITS sequence data from 15 species of Garcinia*. Figures are tree length based on the numbers of DNA substitution changes. Key: O-*Clusia *outgroup Xn-Section* Xanthochymus *Gr-Section* Garcinia *Br-Section* Brindonia *Ds-Section* Discostigma

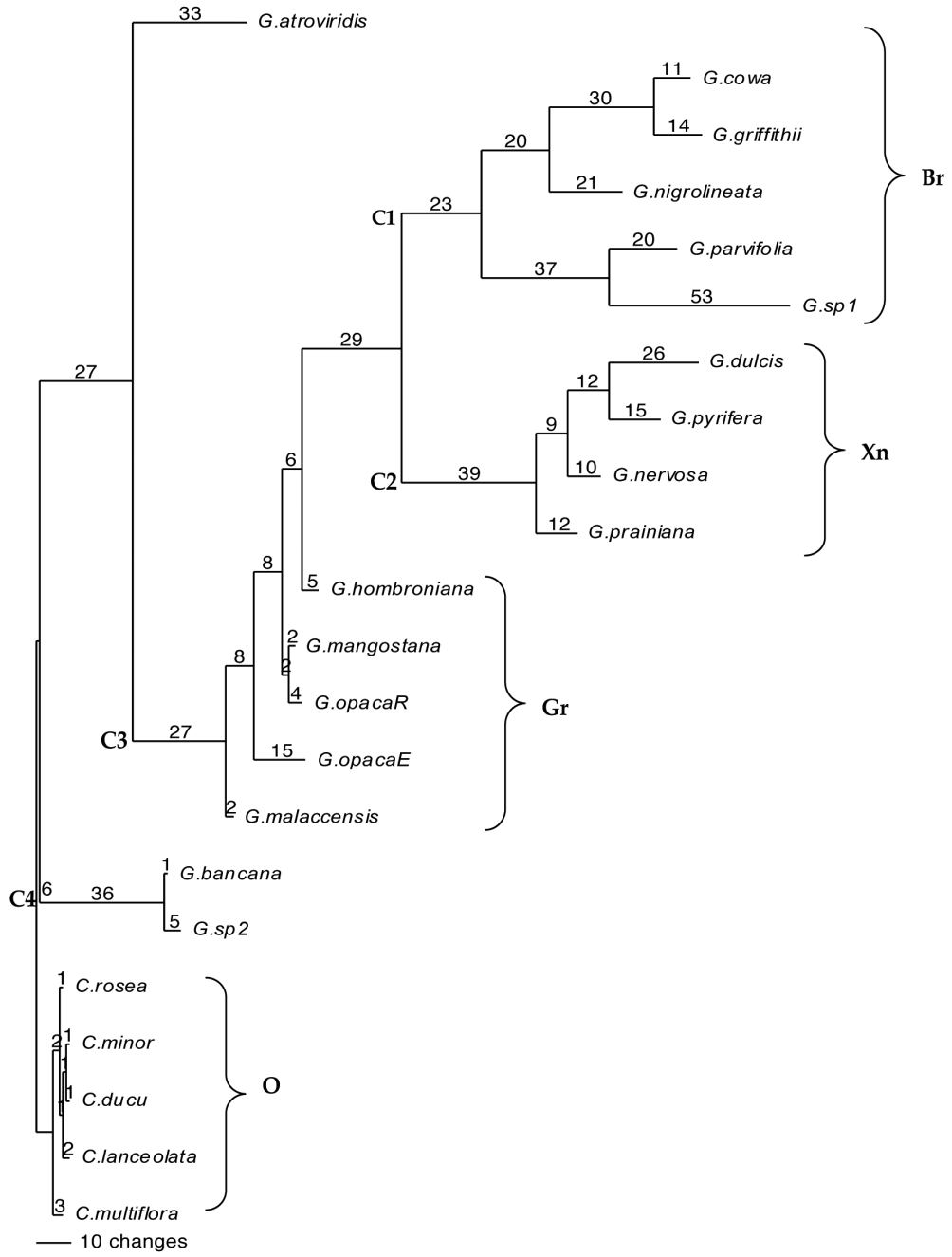

Fig.2: One of the most parsimonious trees based on the trnL sequence data from 17 species of Garcinia*. Figures are tree length based on numbers of DNA substitution changes. Key: O-*Clusia *outgroup Xn-Section* Xanthochymus *Gr-Section* Garcinia *Br-Section* Brindonia

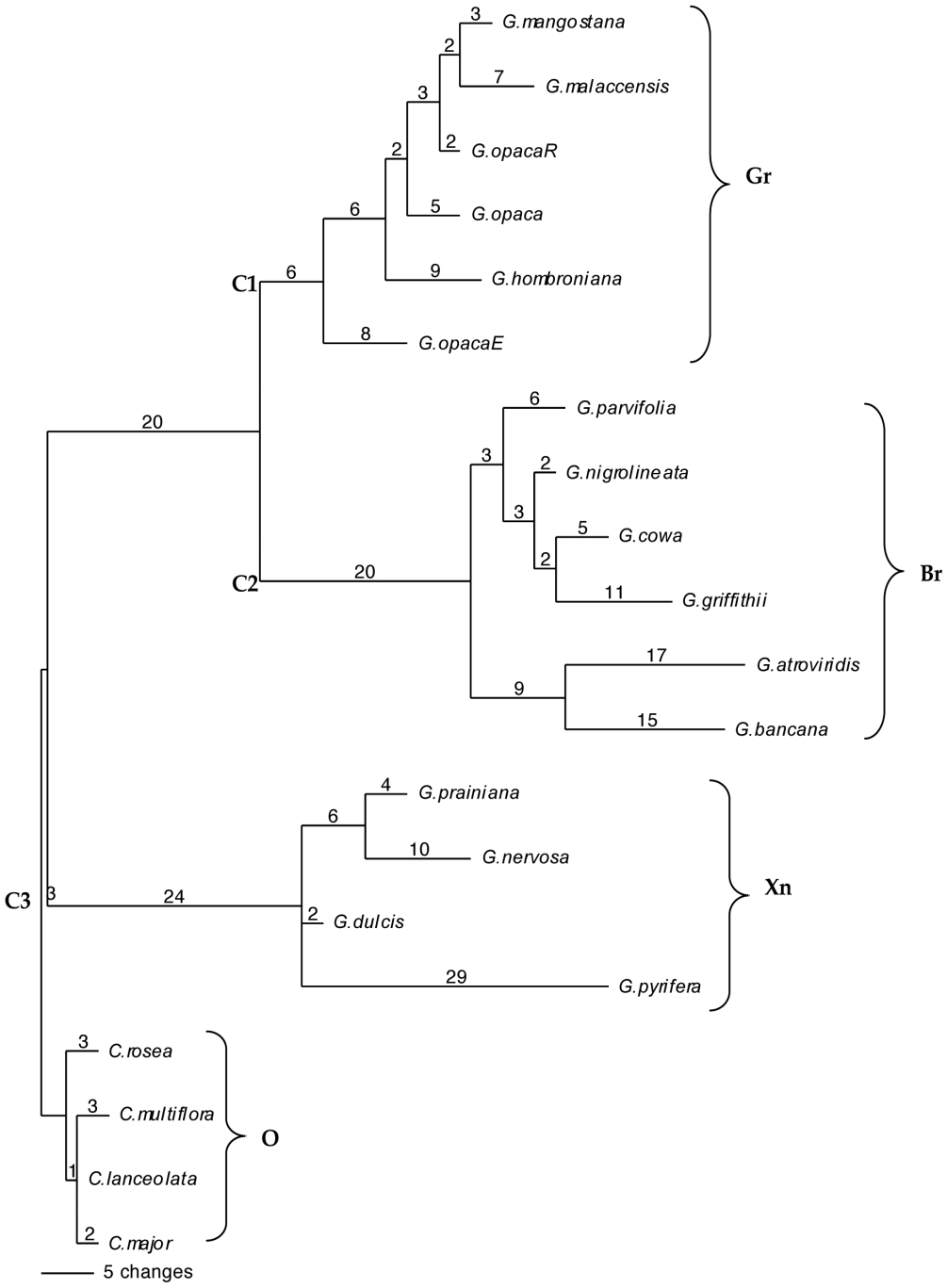

Fig. 3: One of the most parsimonious trees based on the accD-psaL sequence data from 14 species of Garcinia*. Figures are tree length based on the numbers of DNA substitution changes. Key: O-*Clusia *outgroup Xn-Section* Xanthochymus *Gr-Section* Garcinia *Br-Section* Brindonia

bootstrap supported) consists of the species from *Garcinia* section, clade 2 (85% BS supported) is from *Brindonia* section and clade 3 (52% bootstrap supported) is *Xanthochymus* section (*Fig. 3*). In the ITS phylogenetic analysis, *G. bancana* was slightly out of the *Brindonia* section, with 57 nucleotide changes from the main clade and in the trnL region; it is in the same clade with the rest of the members of the Brindonia section but the change of nucleotides was 53, showing that it has distant genetic relationships. In the accD-psaL region, however,

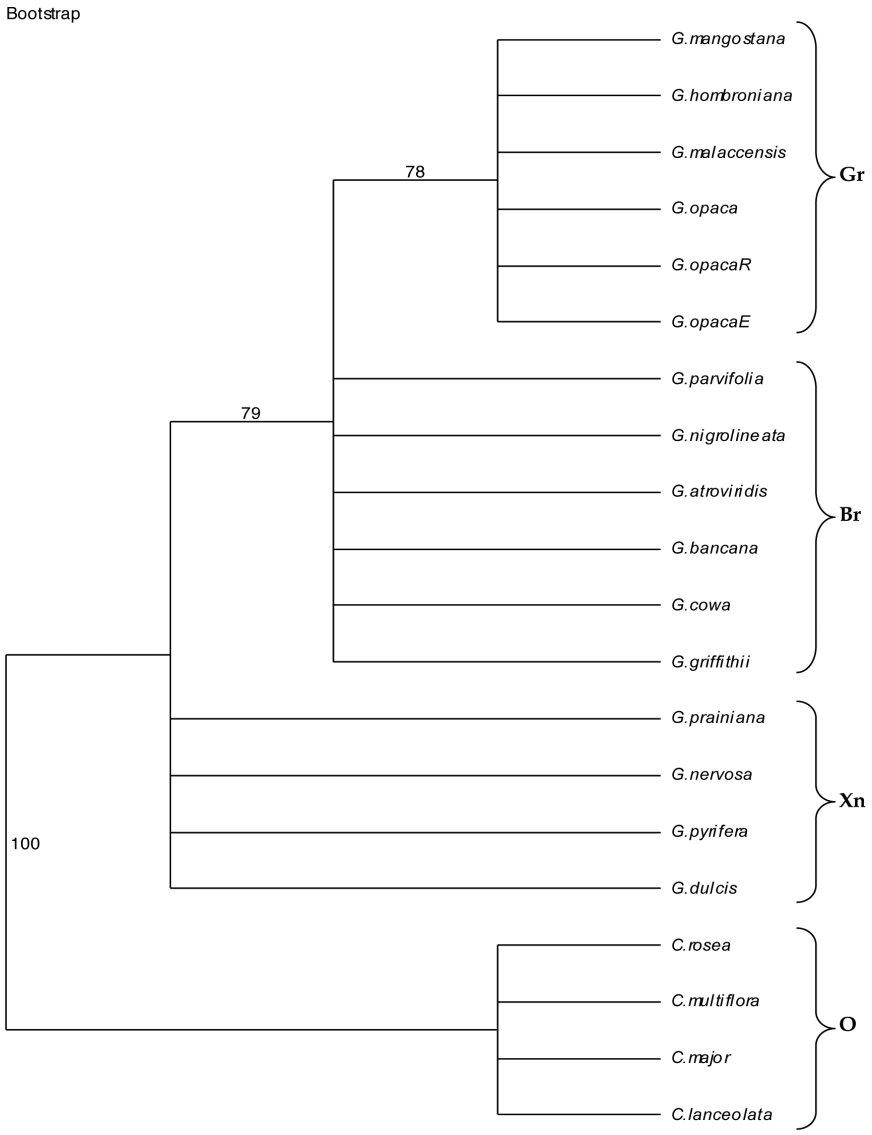

*Fig.4: Bootstrap support (50%) for various nodes (1000 replicates) with the values of more than 78%, based on accD-psaL region. Key: O-*Clusia *outgroup Xn-Section* Xanthochymus *Gr-Section* Garcinia *Br-Section* Brindonia

the changes of nucleotides are only 15 compared to *G. atroviridis*, and this agrees with Jones' (1980) *Garcinia* classification. The number of changes in the branch length between *G. mangostana* with *G. malaccensis, G. opaca, G. opacaE, G. opacaR* and *G. hombroniana* was 10, 8, 11, 5, and 12, respectively. The accD-psaL chloroplast region of *Garcinia* contains mononucleotide repeats of the A nucleotide but the length of the repeats was shorter than the ones in the trnL chloroplast region. The mononucleotides were at 460-470bp with 9–12 repeats. Meanwhile, the primers were able to amplify *G. opaca* DNA but not *Garcinia* sp.1. The number of nucleotide changes was more in the ITS region compared to the trnL, accD and psaL regions. This is typical as the rate of mutation in the internal transcribe spacer regions is much faster than that in the conserved chloroplast regions. Nevertheless, all the three regions showed similar results, whereby *G. mangostana* was found to be more closely related to *G. malaccensis* and *G. opaca* rather than to *G. hombroniana*.

CONCLUSIONS

The three molecular markers proved that *G. hombroniana*, which has previously been suggested as being one of the parents for *G. mangostana*, is unlikely to be so. The samples from three different geographical locations of *G. hombroniana* showed no similar sized microsatellite alleles to *G. mangostana* and *G. malaccensis*. On the other hand, *G. opaca* which was shown by three phylogenetic tree generated from the sequence of the ITS, trnL and accD-psaL regions showed close relationships to *G. mangostana* and *G. malaccensis*. *G. opaca* also shared similar sized alleles to the two species. In all the phylogenetic analyses, *G. hombroniana* has a distant relationship with *G. mangostana* compared *to G. malaccensis* and *G. opaca*. This strongly suggests that one of the parents for *G. mangostana* is *G. malaccensis* and another possible parent is *G. opaca*, but not *G. hombroniana*.

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Phylogeny and Phylogeography of *Myotis muricola* (Gray, 1846) (Chiroptera: Vespertilionidae) from the West and East of Wallace's Line Inferred from Partial MtDNA Cytochrome *b* Gene

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ABSTRACT

Myotis muricola is a widespread species covering the Malay Archipelago through the West and East of Wallace's Line. The genetic analysis, based on partial cytochrome *b* gene, shows the high genetic variation within *M. muricola*. The phylogenetic analysis has indicated that *M. muricola* in the Malay Archipelago are monophyletic. Members of *M. muricola* Eastern are grouped together independently of *M. muricola* Western and both groups are distantly related. On the other hand, *M. muricola* Western and *M. muricola* Eastern are distinct species and sister taxa to *M. mystacinus*. Based on the high genetic distance (26.8% to 38.5%) and the Genetic Species Concept (Baker & Bradley, 2006), it can be concluded that *M. muricola* Western and *M. muricola* Eastern should be considered as two distinct species. Furthermore, two subgroups within *M. muricola* Western, namely Sumatra-Asian and Bornean subgroups, are recognised as distinct subspecies (with genetic distance of 5.1% to 10.8%). The evidence from the molecular data indicated *M. muricola* Eastern as the ancestor of *M. muricola* species complex in the Malay Archipelago, which had earlier diverged into the western region during the Pliocene. Meanwhile, the geographical conditions during the Pleistocene had given more chances for fauna to diversify. It was predicted that *M. muricola* diverged in the western part of the Malay Archipelago during the Pleistocene when the sea level dropped and produced some landbridges among the islands in Sundaland. The hypothetical dispersal routes of *M. muricola* are related to the ancient Sunda River systems that produced gallery forest corridors for migration and which served as Pleistocene refuges during the migration.

Keywords: Chiroptera, *Myotis muricola*, Phylogeny, Phylogeography, cytochrome *b*, Malay Archipelago

INTRODUCTION

The Malay Archipelago region consists of many islands which have the richest biodiversity in the world (Myers *et al.,* 2000). It has many features that tremendously contribute to evolutionary biology studies, particularly the linear island geography which provides natural boundaries to population ranges and has environmental gradients (Maharadatunkamsi *et al*., 2000). Furthermore, the region is one with the gradual physical transitions, typified by a decline in

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rainfall from the west to east (Oldeman *et al.,* 1980), and it has a lot of barriers such as sea. In addition, this archipelago was divided by Wallace's Line into two regions, namely the West and East of Wallace's Line. In the northern part, this line lies between Borneo and Sulawesi Islands. Meanwhile, the Lombok Strait represents the Wallace's Line that divides Bali Island as a part of the western region and Lombok Island as a part of the eastern region in the southern part. The archipelago has an unstable and complicated geological history, experiencing marked changes in the recent past, and a cline in many environmental variables that exists from the west to east, along the archipelago which may influence the distribution, habitat and genetics of its faunal elements (Mayr, 1976). These conditions are associated with rapid speciation processes (Whitmore, 1987) and promote high genetic variations among the species in this region. Some previous studies on bats in this region have revealed that there is a strong gradual cline in genetic variations, such as *Cynopterus nusatenggara* (Schmitt *et al.*, 1995), *Eonycteris spelaea* (Hisheh *et al.*, 1998), *Rhinolophus affinis* (Maharadatunkamsi *et al.*, 2000), two Vespertilionid bats (Hisheh *et al.*, 2004), Macroglossinae species (Jayaraj, 2008) and Malaysian *Kerivoula* (Faisal, 2008; Hasan, 2008).

Myotis muricola is a widespread species that is known from Southeast Asia, from East India to South China, and Indonesia, as well as from Sumatra through the Moluccas and the Philippines (Corbet & Hill, 1992; Suyanto, 2001; Simmons, 2005; Francis, 2008). Taxonomically, based on morphological similarities, *M. muricola* was first described as *Vespertilio muricola* by Hodgson in 1841 (*nomen nudum*), and then by Gray in 1846, with the same name. Meanwhile, Chasen (1940) called most Sundanesian specimens as *M. m. muricola* but employed *niasensis* as a valid subspecies of *M. muricola* for those collected from the Nias Island. Tate (1941), however, suggested that all should be referred to as *M. mystacinus* for the specimens from Sumatra, Java, Borneo, and allocated *niasensis* as a subspecies known as *M. mystacinus niasensis*. Laurie and Hill (1954) followed the lead by Tate (1941) in employing *M. mystacinus* but used *muricola* as the sub-specific name for the specimens from the Lesser Sunda Islands and by implication from much of Sundanesia. Corbet (1978) suggested that *M. mystacinus* and *M. muricola* should be considered specifically distinct. According to van Strien (1986), there are three sub-species of *M. muricola* in Indonesia, and these are *M. m. muricola* (Sumatra, Sipora, Java, Bali, Borneo, Sumba, Sumbawa and Flores), *M. m. browni* (Central Sulawesi) and *M. m. niasensis* (Nias Island). Recently, the classification by Simmons (2005) outlines that *M. muricola* as a distinct species from *M. mystacinus*. It is important to note that *M. mystacinus* does not extent eastwards into Asia beyond Kazakhtan, Syria and Israel. This taxon has been replaced by *M. nipalensis* which is distributed from Iran, Turkey, and Uzbekistan to Nepal, Mongolia, Tibet, NW China and Siberia. Furthermore, the specimen from Vietnam which had originally identified as *M. mystacinus* might represent as *M. muricola* (*see* Bates *et al.*, 1999). On the other hand, Bates *et al.* (2005) recorded *M. mystacinus* from Myanmar as the first authenticated one for Southeast Asia. Therefore, comprehensive studies on *Myotis* are very much needed.

The widely distributed species in the Malay Archipelago shows a genetic structuring that is associated with geography (Hisheh *et al.*, 2004). Previous systematic studies on *M. muricola* have been based on morphological characters, but the systematic status of this species is still unstable and represented as species complex (Francis, 2008). Meanwhile, Faisal (2008) has suggested that there are more than one species under this name, based on a genetic study. Unfortunately, there is no detailed genetic study on *M. muricola* in the Malay Archipelago. Only one genetic study of *M. muricola* has been recorded for Kalimantan and the Lesser Sunda Islands and this was done by Hisheh *et al.* (2004) using allozymes that showed strong associations between genetic and geographical parameters and overall greater genetic variability as measured by mean island heterozygosities.

Over the last two decades, a broad-based DNA sequence data set has become available to examine the biodiversity and speciation in mammals (Baker & Bradley, 2006). Systematicists began to use DNA sequences to study the phylogenetic relationships among taxa, whereas population biologists began to evaluate phylogeographic patterns in DNA sequence variation among individuals within a single species (Avise, 2000). Mitochondrial DNA (mtDNA) offers a particularly valuable source of markers for the study of closely related taxa (Funk & Omland, 2003) because mtDNA evolves rapidly in population of higher animals and it is usually transmitted maternally without intermolecular recombination (Avise, 1998). The cytochrome *b* (cyt *b*) gene has been used in numerous studies of phylogenetic relationships within mammals and its sequence variability makes it the most useful for the comparison of species. Although Galtier *et al.* (2009) have suggested that mtDNA is perhaps intrinsically the worst population genetic and phylogenetic molecular marker, the authors agree that this particular issue is still under constant debate. Therefore, this paper follows some previous studies by Ruedi and Mayer (2001), Piagio *et al*. (2002), Kawai *et al*. (3003), Baker and Bradley (2006), Stadelmann *et al.* (2007), Garcia-Mudara *et al.* (2009), in which cytochrome *b* is stated as a useful marker for molecular study of mammals, especially in bats.

Thus, the aim of this study was to examine the phylogenetic relationships within *M. muricola*, between *M. muricola* and *M. mystacinus* and resolve their taxonomic status using the partial mitochondrial DNA cytochrome *b* gene sequences. Furthermore, this study was

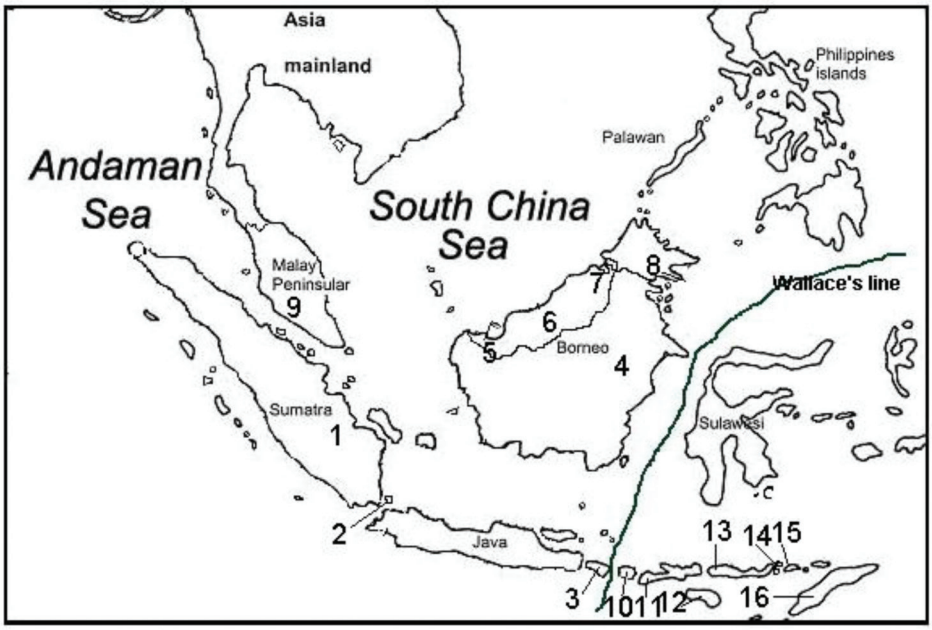

Fig. 1: The localities of the specimens of M. muricola *that were examined. The west of Wallace's Line region is represented by (1) Jambi (2) Krakatau Island (3) Bali Island (4) East Kalimantan (5) Southern Sarawak (6) Central Sarawak (7) Northern Sarawak (8) Tawau, Sabah (9) Fraser Hill, Pahang. The sites from the east of Wallace's Line include: (10) Lombok Island (11) Sumbawa Island (12) Sumba Island (13) Flores Island (14) Lembata Island (15) Pantar Island (16) Timor Island*

also aimed to reconstruct the phylogeography of *M. muricola* species complex in the Malay Archipelago, based on their genetic analyses.

MATERIALS AND METHODS

Study Area

The study area covered the localities from many islands that represented the two different regions, namely, the West and East of Wallace's Line. This study area was divided politically into two countries, with a bigger part of these sampling sites being from Indonesia and the remaining sites were chosen from Malaysia. These sampling sites were chosen based on the distributions of *M. muricola* in the Malay Archipelago. Representative samples from throughout the geographic range, including small islands, were needed to examine the genetic variations of the populations as well as the biogeography of *M. muricola*. Generally, this study was based on the available museum collections. However, some fieldworks were also conducted to cover the geographic range of this particular species. The sampling sites of this study are shown in *Fig. 1*.

DNA Samples and Other Sequence Resources

Genetic materials (liver and tissue) of *M. muricola* were taken from the specimen collections in Museum Zoologicum Bogoriense (MZB), Museum Zoological Universiti Malaysia Sarawak (MZU) and additional fieldwork to acquire the fresh samples in Sarawak and Peninsular Malaysia (Table 1). All of the specimens from the museum and fieldwork were re-identified. The identification of the specimens were according to the identification key by Corbet and Hill (1992), Suyanto (2001), Yasuma *et al.* (2003) and Francis (2008). In particular, *M. muricola* was classified into the sub-genus *Selysius* which differed from the other sub-genus by having small feet (including claw < ½ of the tibia length) with the wing membrane that usually is attached at the base of the toes (Corbet & Hill, 1992; Yasuma *et al.*, 2003). Furthermore, *M. muricola* differs from the other species within the subgenus *Selysius* by having upper part brown to grey with dark bases, under parts with dark bases, and light brown tips. Ears are moderately long, tragus slender, bent forwards and bluntly pointed. The feet are small with wing membranes attached at the base of the toes (Francis, 2008). The length of the forearm ranges from 30-37 mm with three pairs of premolar. Braincase is flatter, and it is not rising abruptly from the rostrum. The condylobasal length is 12.7-13.1 mm and condylocanine length is 11.2-12.6 mm. The second upper premolar is small, rarely very small, and is usually in row or slightly intruded. Meanwhile, the second lower premolar is usually in row, and it is sometimes slightly intruded (Corbet & Hill, 1992; Suyanto, 2001; Yasuma *et al.,* 2003). Unfortunately, a lot of specimens from the collections of MZB were unsuccessfully extracted for DNA because they were preserved in formalin as the preservative chemical when these samples were collected during the fieldwork, although they were then transferred into ethanol in the museum. The DNA was already degraded by this chemical. If the DNA was still in the tissue and it was more a matter of getting it out than it being chopped up (White & Densmore, 1992). However, only eight samples of *M. muricola* which had been contaminated by formalin were successfully extracted. Some sequences from the previous studies were included in this study, and these included several sequences of *M. mystacinus* to construct the relationships among the *M. muricola* populations. All of these additional sequences were taken from GenBank (Table 2).

DNA Extraction

Total DNA was extracted using the CTAB (cetyl-tri-methyl ammonium bromide) protocol (Ducroz *et al.*, 1998; Lecompte *et al.*, 2005). 700 µl 2X CTAB buffer and 8 mg of proteinase K were added into the 1 cubic millimetre minced tissue samples to denature the proteins to eliminate the proteins which could affect the DNA product (Di Mito & Betschart, 1998). Then, 700 µl of chloroform-isoamyl alcohol was added to inhibit

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

Species	Museum reference	Location		Habitat	Abbr.	GenBank Accesion No.
		Geographical region	Locality of specimen			
M. muricola	MZB 28109	Southern Sumatra	Jambi	secondary forest	Mm Jambi1 SM	GU358630
M. muricola	MZB 28110	Southern Sumatra	Jambi	secondary forest	Mm Jambi2 SM	GU358631
M. muricola	MZB 28111	Southern Sumatra	Jambi	secondary forest	Mm Jambi3 SM	GU358632
M. muricola	MZB 16999	Krakatau	Krakatau Island	na.	Mm Kr	GU258636
M. muricola	MZB 23629	Eastern Kalimantan	Long Bawan, Kayan Mentarang	agricultural plantation	Mm EK	GU358635
M. muricola	TK 153691*	Peninsular Malaysia	Fraser Hill, Pahang, Malaysia	mixed dipterocarp forest	Mm PahangPM	GU358633
M. muricola	MZU/M/00321	Southern Sabah	Tawau Hill	lowland dipterocarp forest	Mm Sbh	GU358634
M. muricola	MZU/M/00302	Southern Sarawak	Kubah National Park	mixed dipterocarp forest	Mm KubahSWK	GU358641
M. muricola	MZU/M/00303	Southern Sarawak	Kubah National Park	mixed dipterocarp forest	Mm Kubah2SWK	GU358642
M. muricola	MZU/M/00767	Southern Sarawak	Jambusan cave, Bau limestone	secondary forest	Mm BauSWK	GU358639
M. muricola	MZU/M/00504	Southern Sarawak	Mount. Pueh, Sematan	mixed dipterocarp forest	Mm PuehSWK	GU358640
M. muricola	TK 153582*	Central Sarawak	Bukit Lima, Sibu	lowland dipterocarp forest	Mm SibuSWK	GU358643
M. muricola	TK 153649*	Central Sarawak	Menyarin River, Lanjak Entimau	mixed dipterocarp forest	Mm LanjakSWK	GU358644
M. muricola	MZU/M/00559	Central Sarawak	Similajau, Bintulu	lowland dipterocarp forest	Mm SmjSWK	GU358638
M. muricola	MZU/M/01576	Northern Sarawak	Niah National Park	lowland dipterocarp forest	Mm NiahSWK	GU358637
M. muricola	MZB 20009	Western Lesser Sunda	Bali Island	na.	Mm Bali	GU358646
M. muricola	MZB 19931	Western Lesser Sunda	Lombok Island	deciduous forest	Mm Lombok	GU358648
M. muricola	MZB 19979	Central Lesser Sunda	Sumbawa Island	deciduous forest	Mm Sumbawa	GU358651
M. muricola	MZB 19996	Central Lesser Sunda	Sumba Island	deciduous forest	Mm Sumba	GU358645
M. muricola	MZB 20034	Eastern Lesser Sunda	Flores Island	deciduous forest	Mm Flores	GU358647
M. muricola	MZB 20055	Eastern Lesser Sunda	Lembata Island	deciduous forest	Mm Lembata	GU358649
M. muricola	MZB 20049	Eastern Lesser Sunda	Pisang, Pantar Island	deciduous forest	Mm Pantar	GU358650
M. horsfieldii	MZU/M/01599	Northern Sarawak	Niah National Park	lowland dipterocarp forest	*M. horsfieldii*	GU358652

Note: (na.) data were not available, *field number (has not been registered at museum reference)

TABLE 2
Additional mtDNA cytochrome *b* sequences from the GenBank

Species	Locality	Abbr.	GenBank	
			Authors	Accession No.
M. muricola A	Asia mainland	Mm A	Tsytsulina *et al.* (2004)	AY665143
M. muricola B	Asia mainland	Mm B	Tsytsulina *et al.* (2004)	AY665144
M. muricola C	Bam Keng Bit, Laos	Mm C	Stadelmann *et al.* (2004)	AJ841957
M. muricola browni	Mindanau, Philippines	Mm browni	Ruedi and Mayer (2001)	AF376589
M. mystacinus A	Europe	Mmt A	Ibanez *et al.* (2006)	DQ120879
M. mystacinus B	Europe	Mmt B	Ibanez *et al.* (2006)	DQ120880
M. mystacinus C	Europe	Mmt C	Ibanez *et al.* (2006)	DQ120881
M. mystacinus D	Morocco	Mmt D	Garcia-Mudarra *et al.* (2009)	EU360642
M. mystacinus E	Morocco	Mmt E	Garcia-Mudarra *et al.* (2009)	EU360643

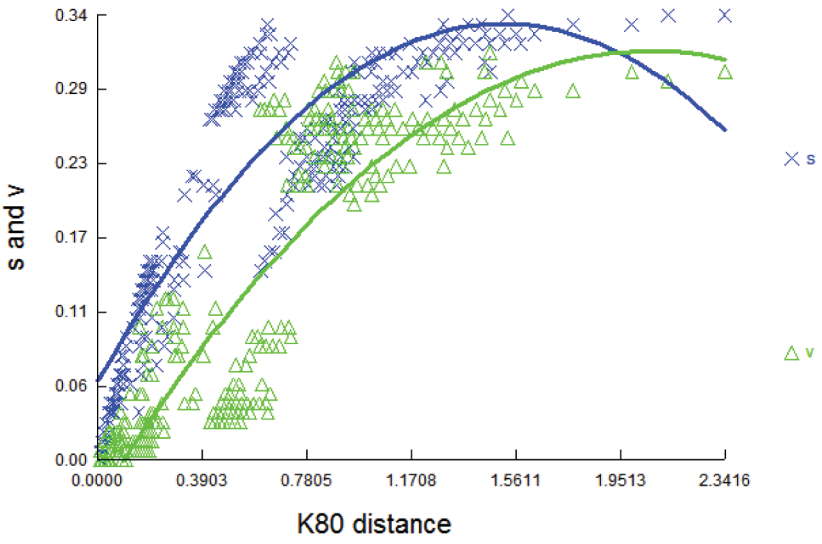

Fig. 2: Saturation plots (the total number of substitution both in the transition and tranversion versus the total amount of uncorrected sequence divergence) calculated using the Kimura two-parameter model (Kimura, 1980)

the lysis process, which was done by proteinase K, while incubating in a water bath at 60°C for 1-3 hours. After centrifugation at 13000 rpm for 10 minutes, 500-550 µl of the upper aqueous layer containing DNA was transferred into a 1.5 ml fresh microcentrifuge tube. Absolute ethanol of the same amount was also added. The absolute ethanol was used to precipitate the DNA product and avoid interference by other products (Gari *et al.,* 2006). After the second centrifugation, the supernatant was removed into a new tube with 600 µl cold 70% ethanol and 25 µl 3M NaCl. Following the third centrifugation, the excess ethanol was discarded and the pellet was air-dried. Finally, the DNA was resuspended in 30 µl of double distilled water (ddH_2O). The extracted DNA samples were kept at -80 ºC in a freezer for later use.

Amplification and Sequencing

25 µl of reaction volume was used for the PCR amplification. This volume included 1 µl of DNA extract, 1.2 µl of each primer pair (10 mM Glud-GL and 10 mM CB2H), 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, and 0.5 µl of *Taq* DNA polymerase with appropriate buffer and ddH_2O to volume. Meanwhile, the thermal profiles of amplifications started with three minutes of denaturation at 93°C, followed by 30 cycles at 93°C (1 min), 47.2°C (1 min 15 s) and 72°C (2 min), with a final extension at 72°C (5 min). To amplify the partial cytochrome *b* gene, the primers used were GludG-L, (5'-TGACCTGAARAACCAYCGTTG-3') and CB2H (5'-CCCTCAGAATGATATTTG TCCTCA-3') (Palumbi *et al.,* 1991). The purification of the PCR product was carried out using the Promega Purification Kit, following the protocol provided by the manufacturer (Wizard® Genomic DNA Purification Kit-Instruction for use of product). The purified PCR products were sequenced by a private laboratory (1st Base, Selangor, Malaysia) using the ABI 3730 Genetic Analyzer.

Phylogenetic Analysis

CHROMAS version 1.45 (MacCarthy, 1996) was used to display the fluorescence nucleotide bases of the DNA sequence for the analysis. Multiple sequence alignments were done by using CLUSTAL X version 1.81 programme (Thompson *et al.*, 1997) and subsequently aligned by eye. Molecular Evolutionary Genetics Analysis (MEGA version 3) (Kumar *et al.,* 2004) was used to perform the analysis of base frequencies and distance matrix. Genetic divergence (Saitou & Nei, 1987) analysis was carried out using Kimura two-parameter model (Kimura, 1980). Meanwhile, the sequence saturation analysis was done using DAMBE version 5.0.7 (Xia & Xie, 2001) by plotting the total number of substitutions against the total amount of uncorrected sequence divergence which was calculated using Kimura's two-parameter model (Kimura, 1980).

Phylogenetic relationship was constructed using four methods; namely, neighbour-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference. The NJ method was implemented using the Kimura two-parameter as the model of evolution (Kimura, 1980), whereas, the weighted MP analysis was done using full heuristic searches, tree-bisection-recognition (TBR) branch-swapping and random stepwise addition. ML inference applied the best-fit maximum likelihood model of sequence evolution using the Akaike information criterion (AIC) in Modeltest 3.7 (Posada & Crandall, 1998). NJ, MP and ML implemented in the Phylogenetic Analysis Using Parsimony (PAUP*) Version 4.0b4 (Swofford, 1998) and the pylogenetic confidence was estimated by bootstrapping (Felsenstien, 1985), with 1000 replicate data sets for the NJ and MP methods, except for the ML method with 100 replicate data sets. Furthermore, the Bayesian inference implemented in MRBAYES 3.0b4 (Ronquist & Huelsenbeck, 2003) was used to estimate a phylogeny by applying different models of molecular evolution for each partition of the molecular data, based on the substitution

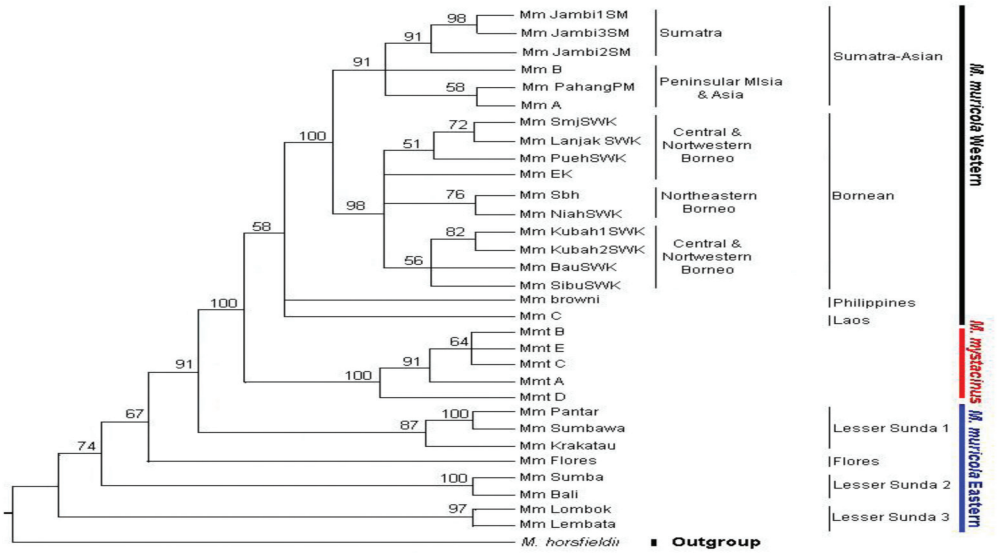

Fig. 3: Neighbour-joining (NJ) tree showing the relationships between M. muricola *vs.* M. mystacinus *and between the groups and sub-groups within the* M. muricola *populations based on the partial cytochrome b mtDNA and calculated using the Kimura two-parameter model of evolution (Kimura, 1980). The values on the branches represent NJ bootstrap values, based on 1000 replicates. Only the bootstrap values >50% are shown. Meanwhile, species abbreviations are given in Tables 1 and 2*

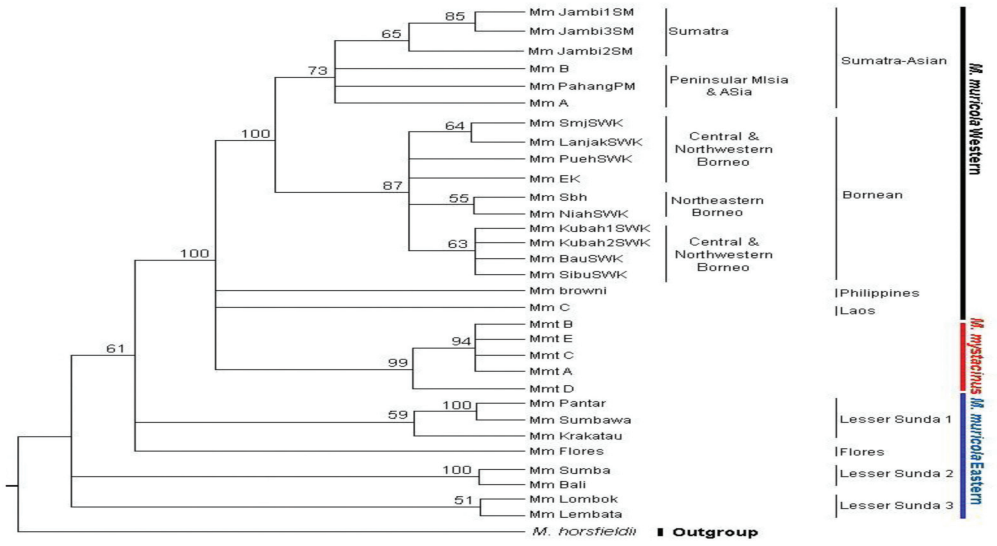

Fig. 4: The weighted and rooted Maximum parsimony (MP) tree showing the relationships between M. muricola *vs.* M. mystacinus *and between the groups and sub-groups within* M. muricola *populations based on the partial cytochrome b mtDNA. The values on the branches represent MP bootstrap values, based on 1000 replicates. Only bootstrap values >50% are shown. Species abbreviations are given in Tables 1 and 2*

model and phylogenetic parameters identified as optimal by AIC criterion in Modeltest 3.7 (Posada & Crandall, 1998). The time of divergence of bats in this study was estimated following (Irwin *et al.*, 1991) the evolutionary rate of cytochrome *b* gene as 0.2% substitution per million years (Myr) and was calculated based on the Kimura two-parameter distance matrix implemented in MEGA version 3 (see above).

RESULTS

Sequences Analysis

A total of 32 partial sequences of 412 bp each of cytochrome *b* were obtained and used in the phylogenetic analysis of this study. These sequences consisted of 26 sequences of *M. muricola*, five sequences of *M. mystacinus* and one sequence of *M. horsfieldii* as an out-group. The average of the nucleotide composition was T (31.0%), A (28.5%), C (24.4%) and G (16.1%). According to Briolay *et al*. (1998) and Bastian *et al*. (2001), the composition of nucleotide showing the anti-G bias is one of the characteristics of mitochondrial DNA. From 412 bp sequences, 200 (48.5%) were variable sites and 152 (76%) were parsimoniously informative sites. Among 31 sequences of *M. muricola* and *M. mystacinus*, 29 haplotypes were identified and 27 were regarded as unique, while two others shared haplotypes within each group; namely, haplotype 1 (Mm Jambi1SM and Mm Jambi3SM) and haplotype 18 (Mmt B and Mmt E). Nonetheless, no haplotype was shared among the groups.

Saturation plots (*Fig. 2*) revealed a high degree of saturation in the third position codon. This was indicated by the fitted curves for transversion having reached the same level as transition. Therefore, the maximum parsimony trees were generated by characters-weighting strategy (with all the characters equally weighted). Furthermore, the maximum likelihood and Bayesian inferences applied the best-fit model of sequence evolution from within the range of models evaluated by script MrAIC (Nylander, 2004) using the Akaike information criterion (AIC). The models that partitioned the rates of change by codon positions were best to account for the sequence characteristics that obscured phylogenetic signal, such as the saturation in substitutions and significant differences in base composition. These models also ameliorated (but did not entirely avoid) the impact of taxon sampling on phylogeny reconstruction (Davalos & Perkins, 2008). Based on the AIC, by using Modeltest 3.7 (Posada & Crandall, 1998), the best-fit model K81uf+G (Kimura, 1981) was applied for the maximum likelihood and Bayesian inferences in this study.

The average pairwise distance (Table 3) was calculated using the Kimura two-parameter model (Kimura, 1980). The pairwise

TABLE 3
Average pairwise genetic distance matrix between the groups and sub-groups calculated using the Kimura two-parameter model (Kimura, 1980)

	M. muricola Western	*M. muricola* Eastern	*M. mystacinus*	Sumatra-Asian
Major group				
M. muricola Western	-			
M. muricola Eastern	0.315	-		
M. mystacinus	0.171	0.26	-	
Sub-group				
Sumatra-Asian	-	0.311	0.168	-
Bornean	-	0.318	0.175	0.072

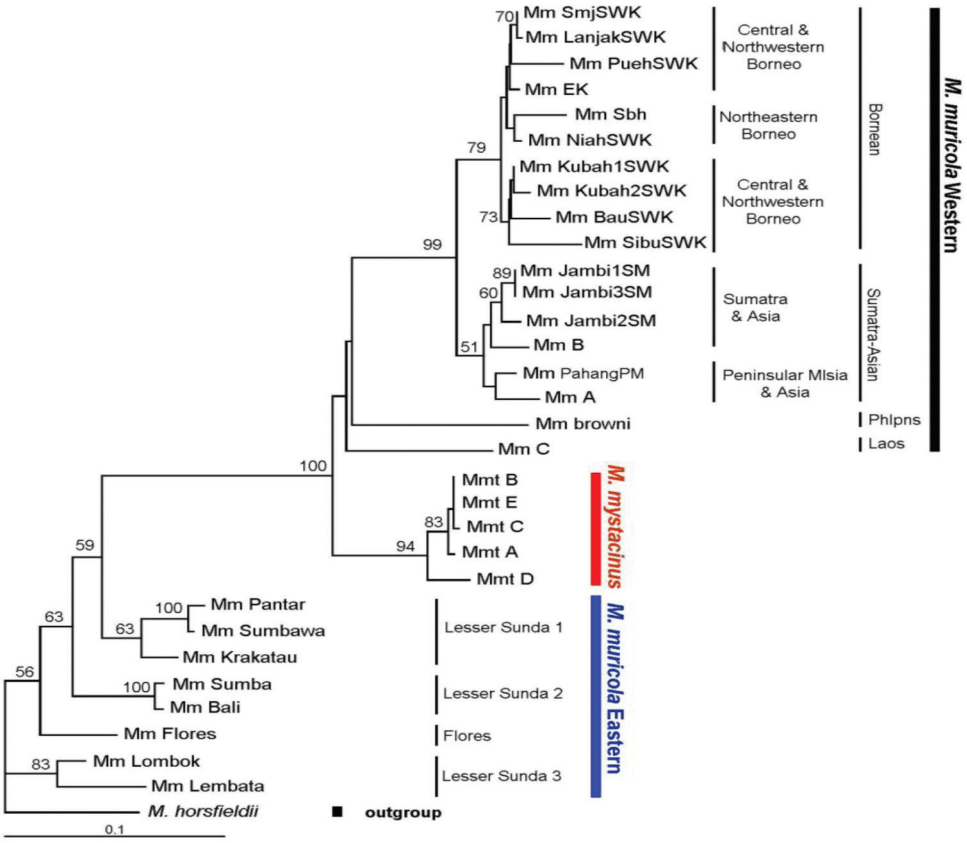

Fig.5: The maximum likelihood (ML) tree showing the relationships between M. muricola *vs.* M. mystacinus *and between the groups and the sub-groups within* M. muricola *populations based on the partial cytochrome* b *mtDNA. The maximum likelihood inference applied the best-fit model of sequence evolution, K81uf+G from the Akaike information criterion (AIC) using Modeltest 3.7 (Posada & Crandall, 1998). The values on the branches represent ML bootstrap values, based on 100 replicates. Only the bootstrap values >50% are shown. Species abbreviations are given in Tables 1 and 2*

comparisons among all the sequences (including the out-group) ranged from 0% to 39.9%. In this study, no sequence from Java Island was available. Generally, all the sequences of *M. muricola* formed two major groups, with the Western group named as *M. muricola* Western consisting of the sequences from Sumatra, Peninsular Malaysia, the Asian mainland (AY665143, AY665144 and AJ841957) and the Philippines with an average genetic distance of 8% (ranging from 0% to 21% in differences within this group). The second group, referred to as *M. muricola* Eastern, was formed by the sequences from Krakatau, Bali, Lombok, Sumba, Sumbawa, Flores, Lembata and Pantar, with an average genetic distance of 9.5% (ranging from 1% to 16.4%). Within *M. mystacinus*, on the other hand, the average genetic distance was 1.5% (ranging from 0% to 3.5%).

The average genetic distance between *M. mystacinus* and *M. muricola* was 17.1%, (ranging from 13.9% to 19.7%, with *M. muricola*

Western) and 26% (ranging from 23.2% to 30.9%, with *M. muricola* Eastern). Within *M. muricola*, among the Western and Eastern, showed a high average genetic distance of 31.5% ranging from 26.8% to 38.5%. Furthermore, within *M. muricola* Western, the specimens from Sumatra, Peninsular Malaysia and mainland Asia formed one sub-group (namely Sumatra-Asian subgroup), whereas the specimens from Borneo became one group (namely Bornean subgroup). The average genetic distance between these two sub-groups is 7.2%, which ranged from 5.1% to 10.8%.

Phylogenetic Analysis

The phylogenetic trees were constructed using NJ, MP, ML and Bayesian inferences, as illustrated in *Fig. 3*, *4*, *5* and *6*, respectively. The maximum parsimony basal clade of the phylogeny was *M. muricola* Eastern, which consisted of *M. muricola* from Lesser Sunda (Bali, Lombok, Sumba, Sumbawa, Pantar, Lembata and Flores) and Krakatau. This particular group diverged from the other two groups around 5.2 Mya. The separation of this group was supported by low bootstrap value in MP, ML and Bayesian (61% for MP, 59% for ML and 57% for Bayesian), but a higher bootstrap value in NJ (91%).

M. muricola Western, which consisted of the sequences from Sumatra, Peninsular Malaysia, Asian mainland, Borneo and Philippines, is a sister species to the *M. mystacinus* group and it diverged around 3.4 Mya. The relationship between these groups is supported with high bootstrap values (100% in all the analyses).

M. muricola Western consisting of the two big sub-groups was fully resolved by using the NJ, MP, ML and Bayesian methods. The branching topology between the two sub-groups was supported by high bootstrap values (100% for NJ, MP, Bayesian and 99% for ML). The Sumatra-Asian sub-group was formed by the sequences from Sumatra, Peninsular Malaysia and two sequences from the Asian mainland (AY661543 and AY661544). On the other hand, the Bornean sub-group consisted of the sequences from the Borneo Island (Sarawak, Sabah and Kalimantan). It is important to note that the separation of these sub-groups was estimated to be around 1.44 Mya.

DISCUSSION

Three major groups were resolved in all of the phylogenetic tree analyses, and based on NJ, MP, ML and Bayesian, the *M. muricola* species formed in the Malay Archipelago are reciprocally monophyletic. At the basal of the phylogenies is *M. muricola* Eastern, which consists of *M. muricola* from Lesser Sunda, which is thus hypothesised as the ancestor of *M. muricola* in the Malay Archipelago. Members of *M. muricola* Eastern are clustered independently of *M. muricola* Western and both the groups are distantly related. On the other hand, all the phylogenies also showed the sister relationship of *M. muricola* Western and *M. mystacinus.*

Phylogenetic Relationship of M. muricola *and the Taxonomic Implications*

Based on the phylogenetic trees, the relationship between *M. muricola* and *M. mystacinus* was fully resolved and supported with high bootstrap value (100% for all the methods) and *M. muricola* Western is a sister species of *M. mystacinus*. Furthermore, *M. mystacinus* also shows a high genetic distance from *M. muricola*. The average genetic distance between *M. mystacinus* and *M. muricola* was found to be 17% (with *M. muricola* Western) and 26% (with *M. muricola* Eastern). According to Bradley and Baker (2006), and based on the variation in the mitochondrial DNA cytochrome *b* gene, the genetic values higher than 11% indicate species recognition. Therefore, there is genetically no doubt to recognise the taxonomic status of *M. muricola* in the Malay Archipelago as a distinct species from *M. mystacinus*.

Phylogenetic analysis revealed that *M. muricola*, which is widely distributed in the Malay Archipelago, consists of two major groups (namely, *M. muricola* Western and *M. muricola* Eastern), with a high genetic

distance between them. The average genetic distance between the two groups was 31.5%, and this ranged from 26.8% to 38.5% in the differences, suggesting species recognition. Within *M. muricola* Western, the population from Borneo was classified into a different sub-group and separated from *M. muricola* in Sumatra, Peninsular Malaysia and Asia mainland. The average genetic distance between the Bornean and Sumatra-Asian sub-groups was 7.2%, i.e. ranging from 5.1% to 10.8%. Based on these data, the two subgroups are genetically

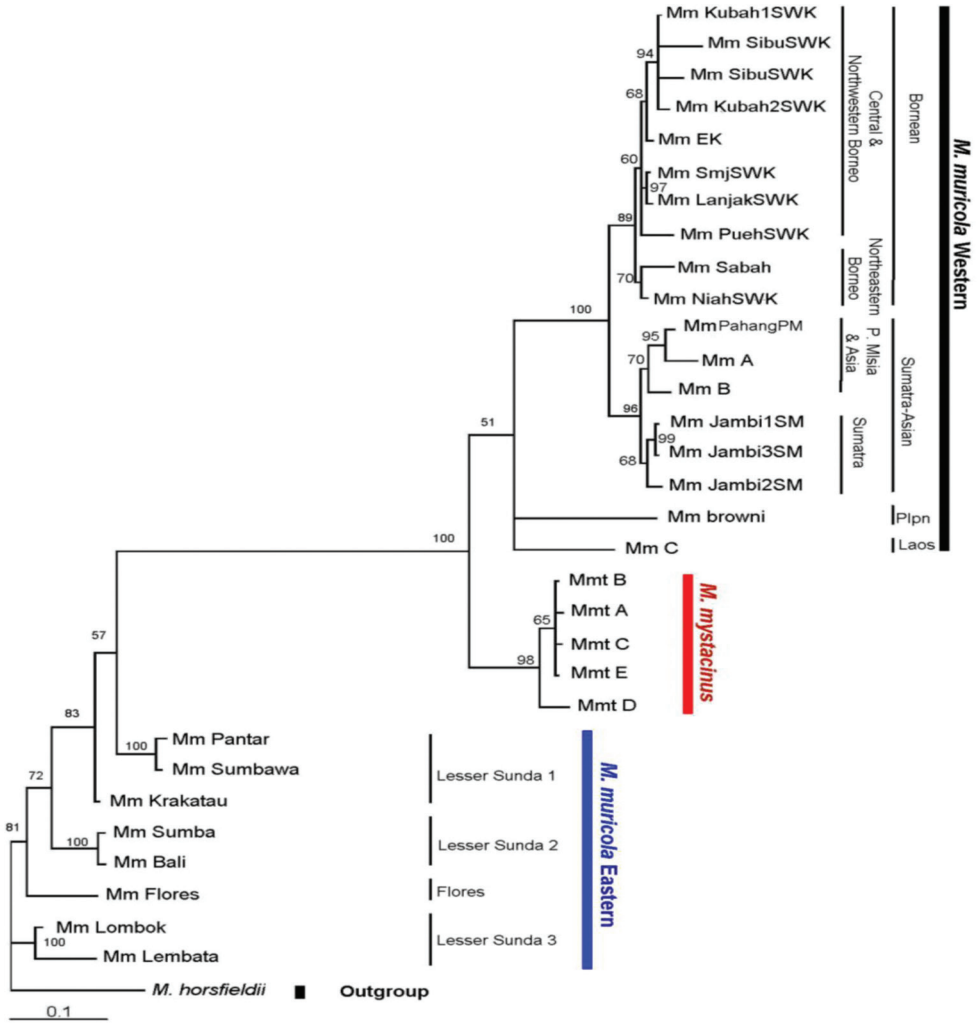

Fig. 6: The Bayesian phylogram showing the relationships between M. muricola *vs.* M. mystacinus *and between the groups and sub-groups within* M. muricola *populations based on the partial cytochrome b mtDNA as implemented in MR BAYES 3.0b4. The values on the branches represent Bayesian bootstrap values. Only the bootstrap values >50% are shown. Species abbreviations are as in Tables 1 and 2*

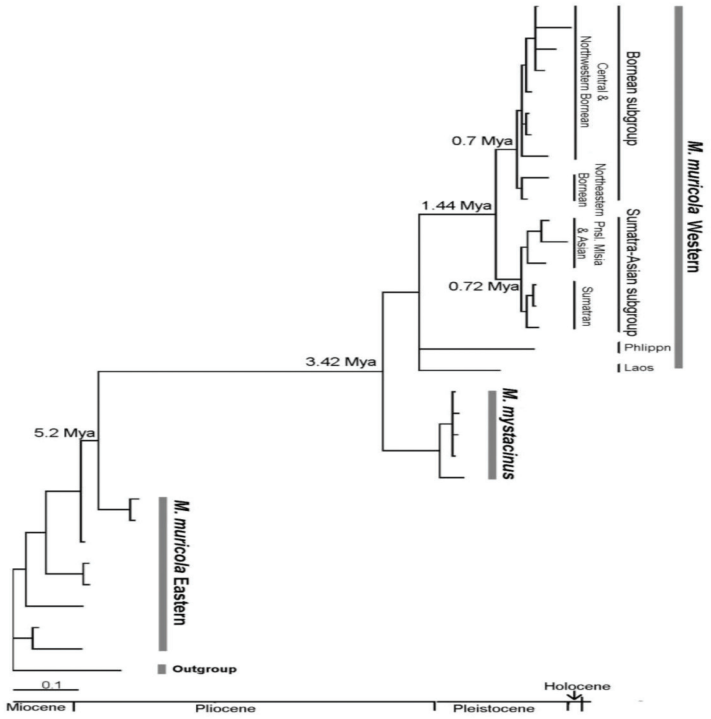

Fig. 7: A chronogram showing the estimated time of the divergence of M. muricola *and* M. mystacinus *obtained from the partial cytochrome b gene. Ages were inferred from the average genetic distance (Irwin et al., 1991) calculated using the Kimura two-parameter model (Kimura, 1980), implemented in MEGA version 3 (Kumar et al., 2004). The Bayesian phylogram is used to show the relationships among these populations*

recognised as different subspecies (Bradley & Baker, 2006). These genetic data become the evidence that the taxonomic status of this species must be resolved. Some new species and sub-species have also been recognised under *M. muricola*.

The Distribution Pattern of M. muricola *in the Malay Archipelago and the Implication of Wallace's Line as a Barrier*

On the basis of genetic, the populations from the larger islands of Greater Sunda (Sumatra and Borneo) and from Peninsular Malaysia formed one group, i.e. *M. muricola* Western. It is generally distinct from the islands in Lesser Sunda (Bali, Lombok, Lembata, Pantar, Flores, Sumba and Sumbawa). Meanwhile, the populations from Lesser Sunda, in combination with Krakatau, were clustered into one group, i.e. *M. muricola* Eastern. The phylogenetic analysis revealed that the two haplotypes from Bali and Krakatau were not clustered into *M. muricola* Western but into *M. muricola* Eastern instead. Geographically, the two localities are located on the west of the Wallace's Line region which is separated by the narrow Lombok Strait (about 23 km wide) as a barrier from the other islands in the east of the Wallace's Line region.

M. muricola Eastern had occurred during the Miocene (before early Pliocene) some 5.2 Mya. The drop in the sea level, however, had not only occurred over the last 2 Mya of the Earth history; the transitions from the early to middle Miocene and from the middle to the late Miocene were marked by the relatively low sea levels (Haq *et*

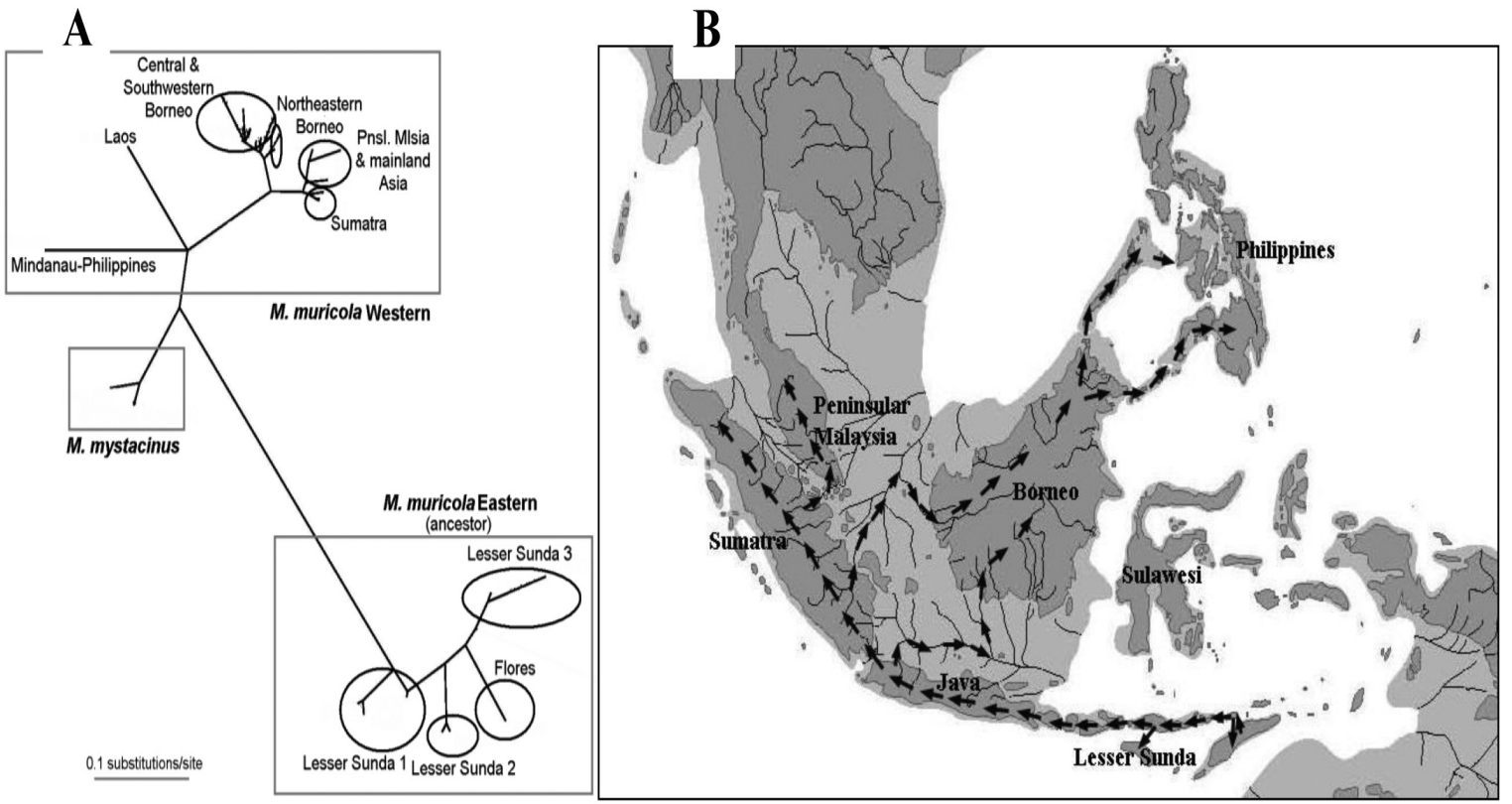

Fig. 8: The hypothetical routes (⟶) of the dispersal of M. muricola *in the Malay Archipelago.* ***A****: Radial tree showing the relationships between* M. muricola *and* M. mystacinus *and within M. muricola in the Malay Archipelago inferred from Bayesian method.* ***B****: Map showing the routes of the dispersal of M. muricola in the Malay Archipelago. Map was adapted or modified from Voris (2000), whereby the sea level was shown to drop 120 m below the present sea level giving rise to some river systems*

al., 1993), and this condition gave more chances for bats with their flight ability to cross the sea barrier, including Lombok Strait. The previous study on *Cynopterus titthaecheilus* showed little genetic differentiation between the bat populations in West Java (west of the Wallace's Line) and Lombok (east of the Wallace's Line) (Kitchener & Maharadatunkamsi, 1991). In addition, Kitchener and Maharadatunkamsi (1991) also noted that the water gap, such as those found between Bali and Lombok islands, did not pose a significant barrier to gene flow in other closely related *Cynopterus* species. Based on these data, this study has also indicated that the drop in the sea level during the Miocene (Haq *et al.,* 1993), as well as in the Lombok Strait, gave the possibility of gene flow within *M. muricola* Eastern, including those from the populations in Krakatau and Bali islands to the other populations in Lesser Sunda.

Sumatra, Borneo and Peninsular Malaysia are an expansion of the Asian continent and these places were connected to each other several times during the Pleistocene period when glaciations lowered the sea level (Heaney, 1991). Based on this genetic study, the samples of *M. muricola* from these islands were grouped into *M. muricola* Western. All the phylogenetic trees revealed that this particular group consisted of two sub-groups, namely, the Sumatra-Asian sub-group (Sumatra, Peninsular Malaysia and Asia mainland) and the Bornean sub-group (from Borneo only), which were supported with high bootstraps value (100% for NJ, MP, Bayesian and 99% for ML). The separation of the two sub-groups occurred during the Pleistocene, some 1.44 Mya. After the Pleistocene, these islands were separated by the Sea of Java and the populations inside these islands were isolated from one to the other island. This isolated population eventually appeared as a sister-group to all the other species in that particular group because any new mutation appearing outside the island did not reach the isolate and only the plesiomorphic characters were shared with the other areas (Ruedi & Fumagali, 1996). Meanwhile, the separation between the two sub-groups within *M. muricola* Western indicated allopatric speciation.

On the other hand, *M. muricola* Eastern has the higher genetic variation compared to the *M. muricola* Western. Based on the phylogenetic trees, members of this particular group did not form one cluster (clade) and thus had high genetic distances ranging from 1% to 16.4% in the difference. *M. muricola* Eastern consists of many islands from Lesser Sunda that were isolated from one another by sea or straits. Furthermore, the minimum contemporary sea crossing between the islands within the Eastern group ranged from four kilometres (between Flores and Lembata) to 117 kilometres (between Bali and Timor) (Hisheh *et al*., 2004). This condition might have led to the genetic differentiation among the populations within this group.

The Origin and Dating of the Diversification of M. muricola

The molecular analyses provide evidence on how the biogeography of *M. muricola* populations in the Malay Archipelago relates to their differentiation and diversification, and the relative timing of these events. Meanwhile, the genetic analyses support the monophyly of *M. muricola* and indicate that the common ancestor of this particular species in the Malay Archipelago is the population in the Lesser Sunda region, i.e. *M. muricola* Eastern. The position of this group, *M. muricola* Eastern, at the basal clade had started during the Miocene and it began to disperse to the western part of the Malay Archipelago during the Pliocene, some 5.2 Mya (*Fig. 7*). Although the sample from Krakatau Island was grouped into the Eastern group, it gave raise to the question in this study and became anomalous. According to Tidemann *et al.* (1990), after the cataclysmic eruption of 1883, the first bat colonisation found on this island some 20-30 years later was by Pteropodids. However, the Microchiropteran had more recent arrivals, probably during recolonising Rakata, i.e. between 50 and 70 years after the eruption. It is predicted that *M.*

muricola from this island are not the ancestor of *M. muricola* in the Malay Archipelago.

Ruedi and Mayer (2001) considered the extent to which paleontological and molecular data could be combined to infer the biogeographic patterns of evolution. The most recent interpretation of the fossil record supports a late Oligocene origin of the genus *Myotis* in Eurasia (Horacek, 2001), and the early appearance of the genus was followed in the early Miocene by burst of diversification, as indicated by the various fossils of *Myotis* found in Europe (Czaplewski, 1991). The evidence of *Myotis* fossils in Asia is fragmentary and more recent (late Pliocene/early Pleistocene), and based on the geographical reconstruction by Teeling *et al.* (2005), it was suggested that the bats originated from the Laurasian land masses which were possibly in North America during the early Paleocene. Nonetheless, the results from this study showed that the common ancestor of *M. muricola* was from Lesser Sunda Island in the Southeast Asia region, and this took place before the Pliocene. Thus, it is proposed that the findings of this study support the Gondwanan origin for the bats with the fossil of *Australonycteris* from Southeastern Queensland, as the evidence suggested that modern bats might have originated from an isolated group of archaic bats in the Southern Hemisphere (Hand, 1994) during the early Eocene. On the other hand, the geological history has also revealed that the animals could have reached the Malay Archipelago from Gondwanaland via Australia during the Miocene (Whitmore, 1981).

M. muricola Eastern diverged to the western part of the Malay Archipelago during the late Miocene to Pliocene, some 5.2 Mya. Cooling and aridification of habitats that occurred during the late Miocene (Cerling *et al.,* 1997) could have triggered the early diversification of bats. In the late Pliocene (some 3.4 Mya), *M. muricola* Eastern diverged into *M. muricola* Western. The genetic variations have proven that *M. muricola* Western is distinct from the common ancestor (i.e. *M. muricola* Eastern) and should be considered as a different species. During the Pleistocene (some 1.4 Mya), *M. muricola* Western was differentiated into two sub-groups, namely, the Sumatra-Asian and the Bornean sub-groups. During the glacial maximum, i.e. when the sea level was lowered to 120 m below that of the present, all the land areas of Sundaland were connected into one large land mass (Verstappen, 1975; Voris, 2000; Bird *et al*., 2005). At that time, many species could freely move through the moist ancient river systems that provided forest corridors in Sundaland, Sumatra, Peninsular Malaysia, Java and Borneo (Heaney, 1985; Voris, 2000).

Surprisingly, the present genetic study has suggested the sister relationship between *M. muricola* Western and *M. mystacinus*. Some of the previous studies have recorded that *M. mystacinus* is distributed from Europe through Nepal and India (Corbet & Hill, 1982; Francis, 2008), whereas Simmons (2005) noted that this particular species was distributed in Ireland and Scandinavia to Russia and the Ural Moutains, Kazakhstan, south of Syria, Israel, and Morocco, but not in Southeast Asia. On the other hand, the recent record of this particular species in Myanmar by Bates *et al*. (2005) is the first authenticated record from Southeast Asia. This study has indicated that *M. mystacinus* is also distributed in the Western part of the Malay Archipelago and this is sympatric with *M. muricola* Western.

These results provide the evidence for the evolution in *M. muricola*, including the origin and time of divergence. Meanwhile, a careful combination of climatic, fossil and genetic evidence can provide a much clearer picture of the evolution (Hewitt, 2000). However, the lack of samples is the limitation to further analysis. Moreover, complete molecular data sets and fossil data are needed for higher level analysis of the evolution of *M. muricola* to confirm the findings of this study.

Hypothetical Route of the Diversification and Distribution of M. muricola *in the Malay Archipelago*

The hypothetical route of colonisation and the diversification of *M. muricola* started from the common early ancestor in Lesser Sunda (*Fig. 8*). Based on the phylogenetic analysis, *M. muricola* Eastern at the basal clade has been predicted as the common ancestor of *M. muricola* in the Malay Archipelago, which later diverged into the western part of this region during the late Miocene to the early Pliocene. The origin of the ancestor was predicted to be either in Lombok or Lembata Island. The drop in the sea level at that time gave a chance to disperse from this origin to the western part of the Malay Archipelago.

Lesser Sunda was formed by the plate collision between Southeast Asia and Australia about 15 Mya (Hutchison, 1989). The habitat in the eastern part of Lesser Sunda was more arid than its western neighbour and it became unsuitable for some species to live in or survive (Mayr, 1944; Earl of Cranbrook, 2009). At that time, *M. muricola* Eastern migrated westward and reached the Java Island. Otherwise, the other evidence of the pigmy stegodonts (*Stegodon sompeonis*) from Java showed that this particular species had migrated from Laurasia to the Gondwanaland margin in Timor by walking on dry land along the volcanic island arch of Flores-Wetar (Audley-Charles, 1981). This evidence gave rise to the possibility of *M. muricola* Eastern, with flight ability for island hopping to reach Java Island through the opposite migration route of the pigmy stegodont.

Close to the Pleistocene (some 1.4 Mya), *M. muricola* colonised and diversified in Sumatra and continued into Peninsular Malaysia and Borneo. The estimation showed that the Pliocene-Pleistocene epoch was the period when the *M. muricola* species complex in Sundaland might have diversified. During the Pleistocene epoch, it was also observed that *M. muricola* divergence is similar to the diversification of the *Kerivoula* species in Sundaland (Faisal, 2008). The result of this study is also in a good agreement with the finding on other taxa by Inger and Voris (2001) who reported that the cladogenic speciation of frogs and snakes occurred in Sundaland over 1 Mya. The lowering of the sea level during the Pleistocene produced a number of land bridges. Sumatra, Java and Borneo were intermittently connected by the exposed Sunda Shelf. Some ancient river systems at that time served as the routes for migration (Voris, 2000; Bird *et al.*, 2005; Earl of Cranbrook, 2009), and possibly as the Pleistocene refuges of fauna as well. *M. muricola* diverged from Java to the Asian mainland through the Sumatra Island. Java was connected to Sumatra when the sea level dropped 50 m below the present sea level (Bird *et al.,* 2005). From Sumatra, *M. muricola* species complex diverged to Peninsular Malaysia through the Malacca Strait River System that connected these two localities. On the other hand, *M. muricola* in Borneo was suggested to have been migrated from Java through the two routes. Firstly, from Java, this species diverged into Sumatra and then reached Borneo through the North Sunda River System (Voris, 2000) via Bangka, Belitung and Karimata islands. The second route was through the East Sunda River System (Voris, 2000) which connected the northern coast of Java Island to the south coast of Borneo.

Meanwhile, the zoogeographic evidence suggested that at the Pliocene and early Pleistocene, there was a land bridge connecting the Philippines to Borneo (Heaney, 1985). This study speculated that this land bridge would be the probable route for *M. muricola* to colonise the Philippines, and thereafter, the population became isolated and adapted to the new habitat resulting it to diverge into *M. muricola browni*.

The geological history and the condition of the ecosystem are among the factors influencing the distribution patterns, as well as the morphological and genetic variations of the widespread species (Mayr, 1976). There was an increase in the climatic oscillations during the Pleistocene in all parts of the world, and thus, it was necessary to colonise, adapt or go extinct; the present lineages had the ability and luck to survive such environmental shifts (Hewitt, 2000). During the Pleistocene, Java Island was

divided into two vegetation types. East Java had more open vegetation type compared to West Java which was dominated by savanna (Bird *et al.,* 2005). Based on these data, it was speculated in this study that some populations of *M. muricola* in East Java had moved and colonised westwards, while the remaining populations adapted to these conditions.

The Bornean *M. muricola* subgroup diverged into two populations, namely, Central and Northwestern Borneo and Northeastern Borneo during the Pleistocene (*Fig. 5.1* and *5.2*). From this evidence, it was speculated that the habitat conditions at that time influenced the genetic variations within the Bornean sub-group. Among other, Meijard (2003) suggested that the north-western part of Borneo remained forested even during the last glacial period which could be suitable sites as Pleistocene refugia for fauna. Earl of Cranbrook (2000) postulated several areas as the probable Pleistocene refuges for the fauna in Northern Borneo, whereas Eastern Borneo was covered with open vegetation types like tree savanna or open deciduous forest (Meijard, 2003).

During the post Pleistocene geographical conditions, the present sea level gave raise to a lot of physical barriers for the fauna to easily migrate to the other islands. Moreover, there were no land bridges connecting the islands within the Sundaland region and within Lesser Sunda Islands. Thus, it was suggested that the barrier had caused geographical and reproductive isolations as well as gene flow obstacles that further led to allopatric speciation. Therefore, the recent study recorded the morphological and genetic variations of the recent populations of *M. muricola* that are related to the biogeography of this particular species in the Malay Archipelago.

CONCLUSION

Through the genetic analysis, *M. muricola* in the Malay Archipelago has been found to have high biogeographical variations and represent a species complex. Some new species and sub-species have been identified as well. Therefore, *M. muricola* Western and *M. muricola* Eastern should be recognised as two distinct species. Furthermore, the Sumatra-Asian and the Bornean sub-groups represent two different subspecies. In particular, *M. muricola* in the Malay Archipelago is monophyletic with *M. muricola* Eastern at the basal clade that has been hypothesised as the ancestor. Based on the molecular data, *M. muricola* Eastern had started to diverse into the western region during the Pliocene and diverged within the western region during the Pleistocene under the influence of the ancient river systems that had produced gallery forest corridors which could be considered as the Pleistocene refuges. Therefore, the results of this study have indicated that the revision of the taxonomic status and the re-evaluation of the evolutionary and biogeography of *M. muricola* are urgently needed. In addition, the lack of samples limited the analysis carried out in this study. Therefore, complete molecular data, using variable markers and representative samples from throughout the geographic range (specifically from Java and Lesser Sunda) are recommended for further studies so as to get the results firmly established.

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Immunomodulatory Effects of Betulinic Acid Isolation from the Bark of *Melaleuca cajuputi*

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ABSTRACT

Betulinic acid and its derivatives showed cytotoxicity against variety of tumour and cancer cell lines comparable to some clinically used drug. In the present study, the immunomodulatory effects of betulinic acid, isolated from the roots of *Melaleuca Cajuputi,* was studied. Immunomodulatory effect was evaluated by using lymphocytes proliferation assay on mice splenocytes, thymocytes and human peripheral blood mononuclear cells (PBMC), while the cell cycle progression of betulinic acid treated PBMC was also studied by using flow cytometer. The production of human interleukin-2 (IL-2) and human inteleukin-12 (IL-12) cytokines was also assessed using enzyme-linked immunosorbent assay (ELISA). The results showed that betulinic acid was able to stimulate the proliferation of mice thymocytes, splenocytes and human PBMC in a time and dose-dependent manner. Meanwhile, betulinic acid treated immune cells were proliferated well at lower concentration (7.5 µg /mL), but growth inhibition occurred at a higher concentration (30 µg /mL). The findings obtained from the cell cycle analysis exhibited the proliferation effect of betulinic acid on PBMC, whereby 43.66 ± 2.60% and 42.83 ± 2.40% of the cells entered G2/M phase after 24h and 48h, respectively. Moreover, betulinic acid also induced extracellular IL-2 and IL-12 production. This finding demonstrates that betulinic acid acts as an immunomodulatory agent that may be useful in enhancing immune system.

Keywords: Betulinic acid, cytokine, immunomodulation, *Melaleuca cajuputi*, PBMC

INTRODUCTION

Betulinic acid [3β-hydroxy-lup-20(19) lupean-28-carbonic acid] is a lupine-type triterpene which was first isolated in 1948 from the bark of the London plane tree (*Platanus acerifolia*) (Jung & Duclos, 2006). It can also be found abundantly in the bark of white birch (*Betula alba*) or chemically derived from betulin (Csuk *et al*., 2006). Betulin is an abundant naturally occurring triterpene and it is found predominantly in bushes and trees. Both betulin and betulinic acids possess a wide spectrum of biological and pharmacological activities (Alakurtti *et al*., 2006).

Jeremias *et al*. (2004) found that betulinic acid induced specific cell death of more than 75% in the primary glioblastoma multiforme cells *in*

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vitro at substantially higher rate than established cytotoxic drugs vincristine. When betulinic acid was compared for *in vitro* efficiency against several leukaemia cell lines with conventionally used cytotoxic drugs, the acid was found to be more active than 9 out of 10 standard therapeutics (Ehrhardt *et al*., 2004). In fact, betulinic acid also has indicated selective apoptotic activity toward melanoma cells (Pisha *et al*., 1995) as well as the tumour cells of neuroectodermal origin (Fulda *et al*., 1998) and B16 (Liu *et al*., 2004). Betulinic acid induced apoptosis is mediated by a decrease in mitochondrial permeability due to the release of mitochondrial cytochrome c into the cytosol (Fulda & Debatin, 2005), the formation of reactive oxidative species and the activation of crm-A-insentive caspase activity (Wick *et al*., 1999) rather than through a ligand/receptor system. When betulinic acid was used in combination with anticancer drug vincristine, it showed synergistic cytotoxic effect on the growth and metastases of melanoma cells (B16F10) *in vivo* (Sawada *et al*., 2004). Moreover, betulinic acid has shown to be non-toxic up to 500 mg/kg body weight in mice (Pisha *et al*., 1995), as well as also cytotoxic on medullablastoma (Daoy), glioblastoma (A172) and melanoma (Mel-Juso) cancer cells without effecting normal human fibroblast (Fulda & Debatin, 2005).

Besides, betulinic acid was also found to inhibit the replication of human immunodeficiency virus (HIV) (Zhou *et al*., 2004; Hashimoto *et al*., 1997). Several betulinic acid derivatives are also very potent and highly selective inhibitor of HIV-1 depending on their specific side-chain modification of the compounds. These compounds function by inhibiting HIV fusion, or as recently demonstrated, by interfering with a specific step in HIV-1 maturation (Aiken & Chen, 2005). Moreover, betulinic acid was able to activate macrophage to produce two groups of protein mediators for inflammation including interleukin-1β and tumour necrosis factor-α (Yun *et al*., 2003). In addition, betulinic acid also possesses anti-malaria and anti-inflammatory activities. Furthermore, betulinic acid was found to be active against chloroquine sensitive (T9-96 strain) and resistant (K1 strain) *Plasmodium falciparum* (Alakurtti *et al*., 2006).

Undoubtedly, betulinic acid exhibited various pharmacological activities. Although comprehensive tests have been carried out to study the cytotoxic, anti-malaria, antivirus and even immunomodulatory effect (on macrophage) of this compound, not many studies have been addressed the immunomodulatory activity of betulinic acid towards mice and human lymphocyte *in vitro*. Thus, the present study was carried out to investigate the immunomodulatory effect of betulinic acid which had successfully been isolated from the bark of *Melaleuca cajuputi* on mice splenocyte, thymocytes proliferation, human peripheral blood mononuclear cells (PBMC) proliferation, as well as human PBMC cell cycle progression and cytokine (Interleukin 2 and 12) induction.

MATERIALS AND METHODS

Materials

Betulinic acid, extracted from the bark of *Melaleuca cajuputi*, was supplied by Prof. Dr. Faujan Ahmad, from Department of Chemistry, Faculty of Science, Universiti Putra Malaysia. The pure compound of betulinic acid appears as a white solid, with melting point of 295-297°C. It was isolated from *Melaleuca sp.* and was chromatographed on a silica gel column with increasing polarity of chloroform and methanol solvent system (Ahmad *et al.,* 1997). The concentrated compound was dissolved in dimethylsulphoxide (DMSO) (Sigma, USA) to obtain a stock solution of 10 mg/mL. Later, a sub-stock solution of 0.06 mg/mL was prepared by diluting 6 µL of the stock solution into 994 µL serum-free culture medium, RPMI 1640 (Sigma, USA) (note that the percentage of DMSO in the experiment should not exceed 0.5%). Then, the stock and sub-stock solutions were both stored at 4°C.

Concanavalin A (Con A) (Sigma, USA), lipopolysaccharide (LPS) (from *Salmonella enteritidis*) (Sigma, USA) and Pokeweed

mitogen (PWM) (1 mg/mL) were used as a positive control. This commercial immunomodulator was prepared by dissolving with RPMI 1640 (Sigma, USA). Meanwhile, the stock and sub-stock solutions were prepared as above. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), Bovine Serum Albumin (BSA), Tris, dimethyl sulphoxide (DMSO), triton X-100, EDTA, RNase A and propidium iodide (PI) were purchased from Sigma. Foetal bovine serum (FBS) was purchased from PAA, ammonium chloride and sodium citrate from Fisher (UK), Interleukin 2 and Interleukin 12 Instant Enzyme Link Immunosorbent Assay (ELISA) kit from Bender MedSystems, Austria, and Ficoll-Paque Plus from Amersham Biosciences.

Preparation of Mice Splenocytes and Thymocytes Cell Suspensions

Imprinting Control Region (ICR) mice, aged 5-8 weeks old, were used in all the experiments of this study. The animals were purchased from Animal House, the Faculty of Veterinary Medicine, Universiti Putra Malaysia. The animals were housed under standard conditions at 25 ± 2°C and fed with standard pellets and tap water. The mice were avoided from stress or specific control. This work had earlier been approved by Animal Care and Use Committee, Universiti Putra Malaysia (UPM), (Ref: UPM/ FPV/ PS/ 3.2.1.551/ AUP-R2).

The procedure for preparing mice thymus and spleen cell suspensions is quite similar. The mice were killed by cervical dislocation. The thymus (located above the heart) and the spleen (located right behind the liver) were removed and quickly washed with HANKS Balance Salt Solution (HBSS) (Sigma, USA). Then, these thymus and spleen were minced and pressed through a sterile wire mesh screen (80μm) using a rubber syringe plunger. The cell suspension was washed with PBS (Sigma, USA), supplemented with 0.1% BSA (Sigma, USA) and 0.06% sodium citrate (Fisher, UK) (PBS-BSA-SC) and spun down at 200 x g for ten minutes. The additional step in preparing the spleen cell suspension was the splenocytes which were spun down with 5 mL lysis buffer (7.56g ammonium chloride (Fisher, UK) with 2.42g of Tris (Sigma, USA) in 1L distilled water) to lyse the red blood cells. The supernatants were discarded and 4mL of Dulbecco's Modified Eagle Medium (DMEM) (Sigma, USA) with 10% heat inactivated FBS (PAA, Austria) were added. The spleen and thymus pellet was resuspended and cell counting was performed to determine the lymphocyte cell number in the suspensions.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Venous blood (20-25mL) was aseptically collected from 20 healthy donors in preservative free heparin tubes. The blood was diluted with phosphate buffered saline (PBS), pH 7.4 and layered onto Ficoll plus at the ratio of diluted blood with Ficoll 2:1 (Amersham). After centrifugation at 400 x g for 50 min, the lymphocytes were collected at the interface and washed three times with the PBS. The cells were resuspended in DMEM with 10% foetal bovine serum and antibiotics.

MTT Cell Viability Assay

The effects of the compound on the cell proliferation of mice splenocytes, thymocytes and human PBMC were first determined using a colorimetric technique, which is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Yeap *et al*., 2007). Briefly, 100 μL of DMEM media with 10% of FBS was added into the all the wells, except row A in the 96-well plate (TPP, Switzerland). Then, 100 μL of diluted compound at a concentration of 60 μg /mL was added into row A and row B. A series of twofold serial dilution of compound was carried out down from row B until row G. Nonetheless, row H was left untouched and the excess solution (100 μL) was discarded. 100 μL of the cells (mice thymocytes, human PBMC, HL60 or NIH3T3), with the cell

concentration at 4 × 10^5 cells/mL, was then added into all the wells in the 96-well plate and incubated in 37°C, 5% CO_2 and 90% humidity incubator for selected periods of 24, 48 and 72 h. After the corresponding periods (either 24, 48, or 72 h), 20 µL of MTT (Sigma, USA) at 5 mg/mL was added into each well in the 96-well plate and incubated for 4 h in 37°C, 5% CO_2 and 90% humidity incubator. The medium with MTT was removed from every well and 100 µL DMSO (Sigma, USA) was then added into each well to solubilise the formazan crystal and incubated for 20 min in 37°C, 5% CO_2 incubator. Finally, the plate was read at 570 nm using µ Quant ELISA Reader (Bio-Tek Instruments, USA). The results of the compound were compared with the result of ConA (1 µg/mL) and LPS (1 µg/mL) for immunomodulation as a control. Each compound and control was assayed in triplicate for three times. The percentage of proliferation was calculated by using the following equation:

$$\% \text{ Proliferation} = \frac{[\text{OD sample} - \text{OD control}]}{\text{OD control}} \times 100$$

Flow Cytometer Analyses

Flow cytometer was used to support the effect of betulinic acid on human PBMC cell cycle progression (Alitheen *et al*., 2001). PBMC was chosen to evaluate the capability of betulinic acid to stimulate the proliferation of lymphocytes *in vitro*. In this study, PWM was used as a positive control. The active concentration chosen for the betulinic acid and PWM to stimulate the proliferation of PBMC was 30 µg/mL and 50 µg/mL, respectively. In this study, 1 mL of PBMC, with a density of 4 × 10^5 cells/mL, was treated with 1 mL of betulinic acid and PWM, respectively, according to their active concentrations as mentioned above. The treatment was carried out in 6 well plates (Nunc, UK) with the total working volume of 2 mL for each well. The treated cells were then incubated for 24, 48 and 72 h and harvested by centrifugation at 1000 rpm (200 x g) for 10 min. Subsequently, the treated cells were fixed with 80% ethanol at -20°C for 2 h. Then, the cells were spun down and washed twice with PBS pH7.5. The cell pellets were finally dissolved and stained in PBS buffer consisting 0.1% triton X-100 (Sigma, USA), 10 mM EDTA (Sigma, USA), 50 µg/mL RNase (Sigma, USA) and 3 µg/mL propidium iodide (PI) (Sigma, USA). This process was done in the dark because PI is sensitive to light. The cells were then incubated for 30 min in 4°C and analyzed using the COULTER EPICS ALTRA flow cytometer (Beckman Coulter, USA) at the Laboratory of Biologic, Faculty of Veterinary Medicine, UPM, within 24 h.

Cytokine Production of Human Peripheral Blood Mononuclear Cells

The expressions of extracellular IL-2 and IL-12 were performed by using Cytokine Instant Enzyme Link Immunosorbent Assay (ELISA) kit (Bender MedSystems, Austria) (Yeap *et al*., 2007). Briefly, the extracted human PBMC, with a cell concentration at 5 X 10^5 cells /mL, was treated with same volume of compound at 30 µg /mL in 6 well culture plates (TPP, Switzerland). The control cultures, without compound and positive control with Pokeweed Mitogen (50 µg/mL), were prepared simultaneously. The culture was then incubated for respective time periods of 24 hours, 48 hours, and 72 hours. After the corresponding period, the samples were washed and pelleted. 50 µL of the supernatant was added into the strip on the plate of the kit and incubated for three hours by shaking at 200 rpm. Then, the sample was washed and immediately added with 3,3',5,5''-tetramethylbenzidine (TMB) Peroxidase substrate in the dark. Finally, 1M Phosphoric acid stop solution was added and the plate was read at 450 nm and 620 nm as reference wavelength using µ Quant ELISA Reader (Bio-Tek Instruments, USA) at the Animal Tissue Culture Laboratory, FBBS, UPM. The result was compared to the control strip in the kit. Each compound and control was assayed in triplicates. Meanwhile, the data were expressed as pg /mL.

Statistical Analysis

All the experiments were performed in triplicates. The results were analysed by using SPSS version 13 and expressed as mean ± standard error (SE). The differences between the means were evaluated using the ANOVA test (one way), followed by the Duncan test and $P < 0.05$ was taken as statistically significant.

RESULTS

Mitogenic Activity of Betulinic Acid on Mice Thymocytes

Betulinic acid was demonstrated to stimulate the proliferation of mice thymocytes in a time and dose-dependent fashion (*Fig. 1*). The proliferation rate of mice thymocytes treated with betulinic acid was less than that of Con A after 24 and 48 h of incubation period. In fact, betulinic acid has also been shown to inhibit mice thymocytes at a concentration more than 15 µg /mL after 72 h of treatment. These data revealed that betulinic acid was toxic towards mice thymocytes at the concentrations higher than 15 µg /mL. However, it has also been shown to stimulate better proliferation of mice thymocytes at the concentration of 7.5µg /mL throughout the treatment periods. In more specific, betulinic acid demonstrated a better proliferation of mice thymocytes compared to Con A after 72 h of treatment at the concentration of 7.5 µg/mL with the value of 60.3% and 29.3%, respectively. This clearly indicates that betulinic acid has the capacity to stimulate mice thymocytes in a dose-dependent fashion at 24, 48 and 72 hours.

Mitogenic Activity of Betulinic Acid on Mice Splenocytes

As shown in *Fig. 2*, betulinic acid stimulated the proliferation of mice splenocytes in a time and dose-dependent fashion. At 24 h of treatment, the proliferation effects of betulinic acid increased significantly with a concentration up to 14.99% at 30 µg /mL. After 48 h or treatment, however, betulinic acid was found to be toxic

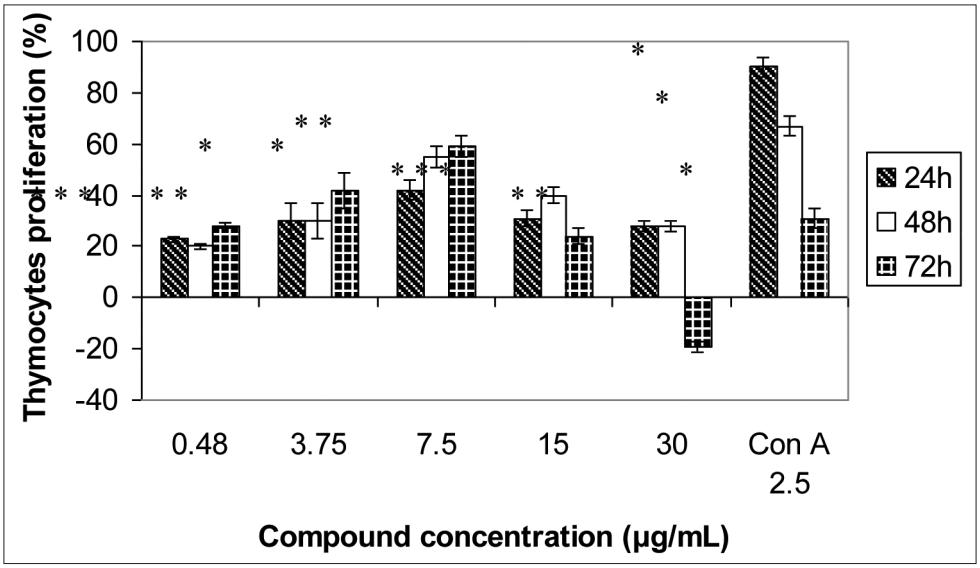

*Fig. 1: The effects of betulinic acid on the proliferative response of mice thymocytes at 24, 48 and 72h of treatments. Data represent the means ± SE of triplicate determinations from three independent experiments. The values were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA (*P < 0.05) and the significant data were marked with **

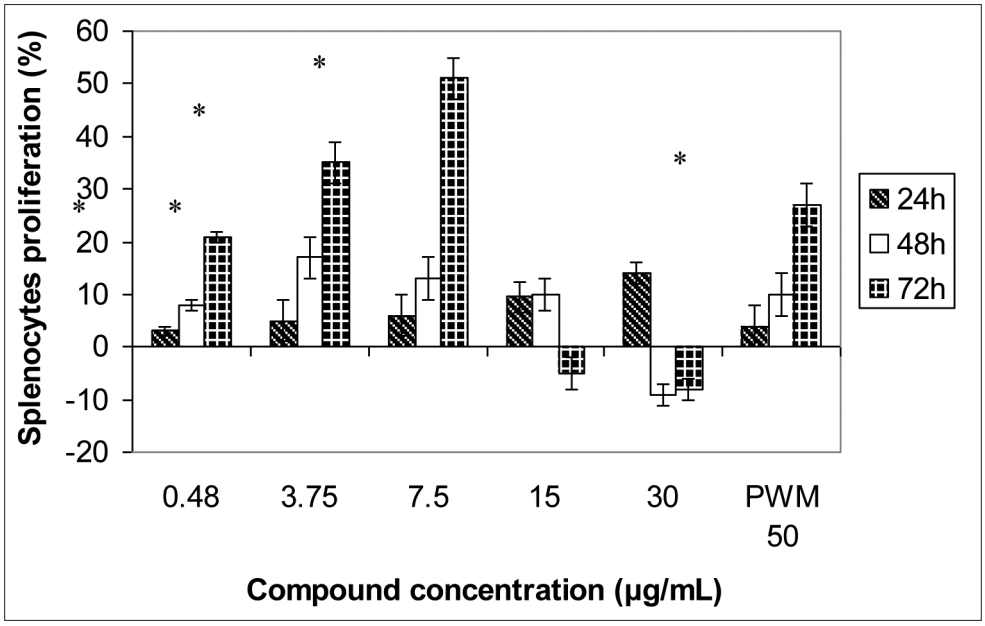

Fig. 2: The effects of betulinic acid on the proliferative response of mice splenocytes at 24, 48 and 72 h of treatment. Data represent the means ± SE of triplicate determinations from three independent experiments. The values were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA ($^{*}P < 0.05$) and the significant data were marked with *

towards mice splenocytes at a concentration higher than 15 µg /mL; nonetheless, it induced proliferation at a concentration lower than that. On the other hand, betulinic acid inhibited mice splenocytes proliferation after 72 h of treatment at a concentration higher than 7.5 µg /mL. Nevertheless, it showed the highest proliferation of mice splenocytes up to 50.77% at this concentration.

Mitogenic Activity of Betulinic Acid on Human Peripheral Blood Mononuclear Cells (PBMC)

Betulinic acid was shown to stimulate the proliferation of PBMC throughout the treatment periods (*Fig. 3*). It did not inhibit PBMC at all the concentrations tested. Meanwhile, the highest proliferation response of the PBMC treated with betulinic acid was 33.72%. Subsequently, the proliferation response of betulinic acid increased significantly with the increase of the concentrations. At 24 h of treatment, betulinic acid exhibited better proliferation of PBMC when compared to the positive control, PWM. However, the proliferation response of the PBMC treated with betulinic acid was less than that of the PWM after 48 and 72 h of treatments. The data obtained showed that betulinic acid stimulated better proliferation of PBMC at 24 h of treatment and was able to sustain for longer incubation time. PWM exhibited a better proliferation of PBMC after 48 and 72 h of incubations.

Flow Cytometry Analysis of Cell Cycle Distributions on PBMC Based on Proliferation Effect of Betulinic acid and PWM at 24, 48 and 72 h of Incubation Time

As illustrated in Table 1, betulinic acid stimulated a higher percentage of cells proliferation at 24 h of treatment compared to the untreated cells that

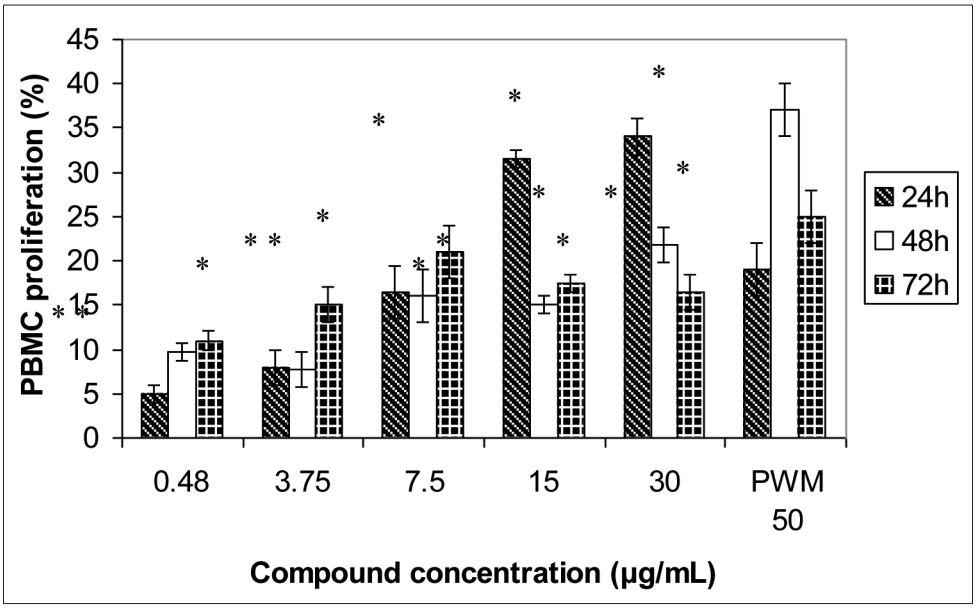

Fig. 3: The effects of betulinic acid on the proliferative response of human peripheral blood mononuclear cells (PBMC) at 24, 48 and 72 h of treatment. Data represent the means ± SE of triplicate determinations from three independent experiments. The values were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA ($^{*}P < 0.05$) and the significant data were marked with *

entered G2/M with value of 45.28% and 4.63%, respectively. This result indicated that the treated cells were 10 folds higher in their cell number compared to the untreated ones. The cells treated with betulinic acid for 48 and 72 h also showed better proliferation with the value of 1.2 folds and 2.5 folds higher, respectively, as compared to the untreated ones. Nonetheless, the percentage of the cells stimulated to proliferate was found to be significantly reduced after 48 and 72 h of treatment with the value of 44.53% and 38.05%, respectively, as compared to those undergoing 24 h of treatment. The reduction of the cells that entered G2/ mitosis corresponded to an increase of cells entering the apoptosis Sub G1 phase. The data indicated that a longer exposure time to betulinic acid might cause apoptosis to PBMC up to 5.76% at 72 h treatment.

The Production of Human Interleukin-2 and Human Interleukin-12

To analyze whether betulinic acid enhanced or suppressed the production of cytokines, the modulatory effect in inducing both human IL-2 and human IL-12 upon stimulation of PBMC was evaluated by using ELISA.

Betulinic acid demonstrated a better induction of human IL-2 after 24 h with 9.6 fold higher compared to the control (*Fig. 4*). This figure showed that it decreased dramatically to 2.5 folds and 1.3 folds, respectively, after 48 and 72 h of the treatment. However, PWM showed a better induction of human IL-2 after 48 h treatment with 25.33 fold higher compared to the control. In addition, it was also demonstrated that there was a stable induction of human IL-2 throughout the treatment period. Betulinic acid was also shown to induce the

TABLE 1
Flow cytometry analysis of cell cycle distribution on PBMCs based on the proliferation effects of betulinic acid and PWM

Compounds	Percentage of cell cycle distribution (%)	Incubation periods (h)		
		24	48	72
Untreated	Sub-G1 (apoptosis)	7.71 ± 0.69	5.59 ± 0.37	1.80 ± 0.03
	G0/G1	5.82 ± 0.52	36.09 ± 2.43	58.71 ± 1.12
	Synthesis	67.25 ± 6.02	24.02 5± 1.59	27.58 ± 0.53
	G2/Mitosis	4.94 ± 0.44	36.59 ± 2.46	15.79 ± 0.30
Betulinic acid	Sub-G1 (apoptosis)	1.43 ± 0.07	3.02 ± 0.16	5.62 ± 0.19
	G0/G1	44.76 ± 1.44*	39.45 ± 2.21	48.18 ± 1.62
	Synthesis	3.59 ± 0.25*	12.95 ± 0.70*	9.05 ± 0.13*
	G2/Mitosis	43.66 ± 2.60*	42.83 ± 2.40	37.12 ± 1.30*
PWM	Sub-G1 (apoptosis)	17.10 ± 2.46*	11.64 ± 2.27*	0.65 ± 1.02
	G0/G1	7.70 ± 1.11	37.53 ± 7.33	39.49 ± 1.11*
	Synthesis	45.11 ± 6.49*	12.04 ± 2.35*	24.02 ± 0.67
	G2/Mitosis	8.40 ± 1.20	26.78 ± 5.23*	41.09 ± 1.15*

The values were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA (*$P < 0.05$) and the significant data were marked with *.

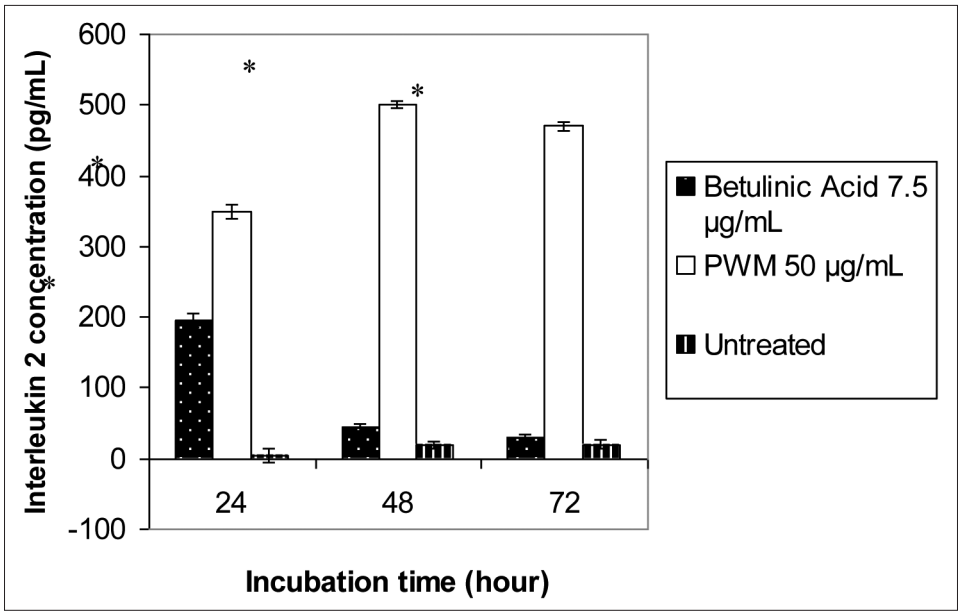

*Fig. 4: The production of human IL-2 in culture supernatants upon the stimulation of PBMC by betulinic acid and PWM. PBMC were isolated and incubated at 24, 48 and 72 h with active concentrations (betulinic acid at 7.5 µg /mL; PWM at 50 µg /mL) and IL-2 induction was specifically determined by ELISA. The values obtained were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA (*P < 0.05) and the significant data were marked with **

production of human IL-2 in a time-dependent fashion. The data obtained also indicated that those compounds had significantly decreased the induction of human IL-2 after 48 and 72 h of incubations. In contrast, the induction of human IL-2 was increased by PWM in a time-dependent manner, which was shown to induce higher production of human IL-2 at 48 h of incubation and decreased slightly after 72 h of the treatment, with the value of 506.66 pg /mL and 466.66 pg /mL, respectively.

In addition, betulinic acid also induced the production of human IL-12 in a time-dependent fashion. It significantly increased the production of human IL-12 from 24, 48 and 72 h with the value of 40 pg /mL, 56.66 pg /mL and 69 pg / mL, respectively, indicating 3.3 folds, 4.72 folds and 5.72 folds higher compared to the negative control (*Fig. 5*). However, the production of human IL-12 induced by LPS decreased sharply after 48 and 72 h, with the value of 26.93 pg / mL or 2.24 folds and 22.06 pg /mL or 1.8 folds higher, respectively, compared to the negative control.

DISCUSSION

The *in vitro* immunomodulatory study showed that betulinic acid was able to stimulate mice thymocytes, mice splenocytes and PBMC in a time and dose-dependent fashion. These results are similar to those of Yun *et al*. (2003), whereby a high concentration of betulinic acid (i.e. more than 10 µg/mL) caused the cytotoxic effect towards mice splenocytes after 72 h of the incubation period. Based on the proliferation effect from both the cells (thymocytes and spleenocytes), it has been clearly shown that betulinic acid is more active towards mice thymocytes as compared to mice splenocytes and

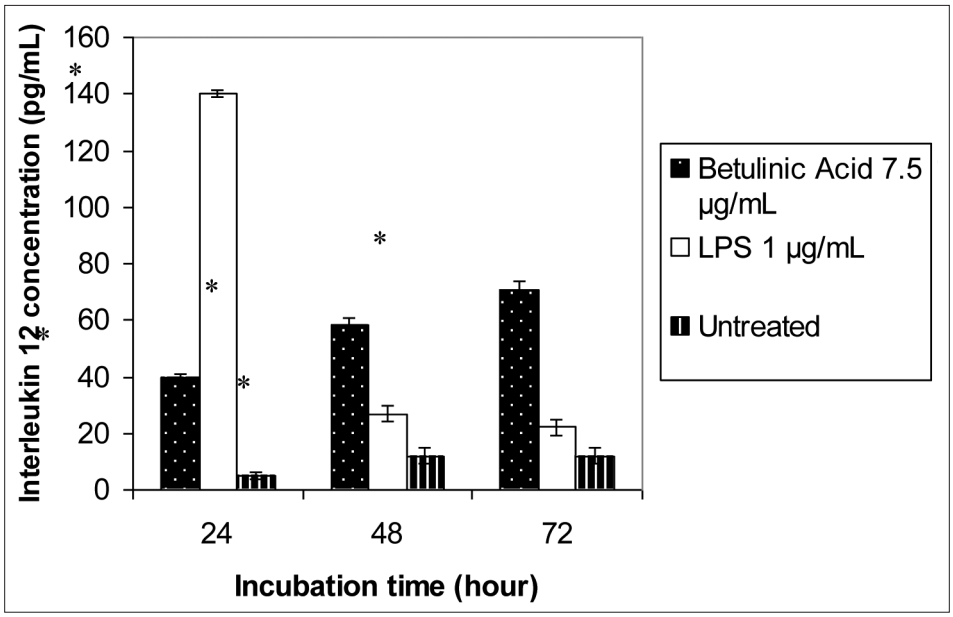

Fig. 5: The production of human interleukin-12 in culture supernatants upon stimulation of PBMC by betulinic acid and LPS. PBMC were isolated and incubated at 24, 48 and 72 h with active concentrations (betulinic acid at 7.5 µg /mL; LPS 1 µg/mL) and IL-12 induction was specifically determined by ELISA. The values were the means ± SE of three experiments. The differences between the control and treated group were determined by one-way ANOVA ($^{*}P < 0.05$) and the significant data were marked with *

a higher proliferation rate at the concentrations of 7.5 µg/mL and above has also been obtained.

Betulinic acid was able to stimulate the proliferation of PBMC without causing cytotoxicity at all the concentrations tested throughout the treatment periods. At 24 and 48 h of treatment, betulinic acid exhibited to be more active at a higher concentration (30 µg /mL) with the proliferation rate of more than 20%. The same pattern of lymphocytes proliferation with mice thymocytes and mice splenocytes could be clearly seen as betulinic acid exhibited to be more active at the concentration of 7.5 µg / mL after 72 h of treatment. This result clearly indicates that betulinic acid tends to induce T cells compared to B cells since the population of T cells in PBMC is about 90% compared to B cells which has only 10% of the PBMC population (Cerqueira *et al*., 2004). This result is also supported by the capability of betulinic acid to stimulate higher production of mice thymocytes (which produce only T cells) and thus supports the preferences of betulinic acid to stimulate T cells compared to B cells.

The cell cycle profile of PBMC treated with betulinic acid was then evaluated by using flow cytometer. In this cell cycle analysis, it was clearly indicated that half of the lymphocytes treated with betulinic acid entered G2 and Mitosis phase (G2/M) after 24 h treatment. However, the percentage of lymphocytes entering the G2/M phase was reduced slowly after 48 and 72 h of treatment, and this was due to the increase in lymphocytes entering the Sub G1 phase. Based on the combination results of the total population increase in the MTT assay and high population of cell under the G2/M phase, it was clearly demonstrated that betulinic acid was able to stimulate the proliferation of human lymphocytes (PBMC) with less toxicity

effect even for a longer incubation time. The result from the cell cycle analysis did support the result from MTT lymphocytes proliferation assay on PBMC. The less toxicity effect of betulinic acid towards normal lymphocytes is in agreement with the previous finding by Zuco *et al*. (2002) who showed that peripheral blood lymphoblasts were resistant against betulinic acid treatment *in vitro*.

Cytokines are soluble glycoproteins which are involved in the immune response activity. The functions of these proteins are diverse, and these include regulating the proliferation and differentiation of lymphocytes (Stanilove *et al*., 2005). A study on cytokines demonstrated that betulinic acid induced the production of human IL-2 and the production of human IL-12 in a time-dependent fashion. Meanwhile, betulinic acid was showed to induce a higher production of human IL-2 after 24 h treatment and the production of IL-2 was reduced dramatically after 48 and 72 h of treatment. The pattern of betulinic acid in inducing the production of IL-2 is quite similar with the pattern of the MTT result of lymphocytes proliferation assay and cell cycle analysis on PBMC, which showed that betulinic acid was more active after 24 h of the treatment. Meanwhile, it could be speculated that the higher induction of IL-2 by betulinic acid was due to the capability of betulinic acid to stimulate the proliferation of T lymphocytes in both the experiments on MTT lymphocytes proliferation assay and cell cycle study. Furthermore, the induction of IL-2 by betulinic acid is relevant to this study since that IL-2 has been known as a central cytokine in the regulation of T cell responses (Ghosh *et al*., 1999). In addition, the populations of T and B lymphocytes in the human peripheral blood lymphocytes, where 90% T cell and only 10% B cell (Cerqueira *et al*., 2004) may contribute to a higher production of human IL-2 in culture supernatant treated with betulinic acid.

In contrast, betulinic acid was demonstrated to induce the production of human IL-12 in a time-dependent fashion. IL-12 is also known as pleiotropic cytokine with important proinflammatory cytokine and immunoregulatory functions. It plays a key role in the modulation of the immune response by providing the stimuli for the differentiation of CD4+ T cells into Th1 and IFN-γ secreting cells. IL-12 is released during the early stages of infections caused by a large variety of bacteria, intracellular pathogens, fungi, and certain viruses. The early production of IL-12 is T cell independent which is caused by direct interaction of pathogens or their products with phagocytic cells (Trinchieri *et al.*, 2003).

The capability of betulinic acid to induce the low concentration of human IL-12 is relevant since this compound has been reported to modulate the Th1/Th2 cells to produce IL-2 and IL-12 cytokines, which are the groups of Th1 as well as IL-10 anti-inflammatory cytokines, which is the group of Th2 (Zdzisińska *et al*., 2003). In a similar experiment, Zdzisińska *et al.* (2003) demonstrated that betulinic acid did not influence TNF-α production, a proinflammatory cytokine. In addition, betulinic acid has also been known to be effective as an anti-inflammatory agent in various experiment systems (e.g. Mukherjee *et al*., 1997; Bernard *et al*., 2001). The capability of betulinic acid to induce small amount of IL-12 production is in agreement with the previous studies which have discovered that betulinic acid is an effective anti-inflammatory agent. Therefore, the evidence indicating that betulinic acid induces low concentrations of IL-12 and TNF-α suggests the involvement of NF-κB signal transduction pathway, which positively regulates proinflammatory cytokine gene expression.

CONCLUSION

Although betulinic acid has shown encouraging results as an immunomodulator, further studies are still needed to elucidate the exact mechanism involved in betulinic acid. Once the mechanism of its action and comprehensive bioassay are elucidated, betulinic acid can be used as a lead molecule for the new generation of drugs in cancer treatment, particularly in boosting up the immune system.

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Phylogenetic Relationships among Several Freshwater Fishes (Family: Cyprinidae) in Malaysia Inferred from Partial Sequencing of the Cytochrome *b* Mitochondrial DNA (mtDNA) Gene

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ABSTRACT

The phylogenetic relationships among 23 species of Malaysian freshwater fishes in the family Cyprinidae was inferred by partial sequencing of the Cytochrome *b* (Cyt *b*) mitochondrial gene. Samples were collected from various localities in Sarawak, Sabah and Peninsular Malaysia. The inferred phylogeny appeared to match major groupings currently recognized in the taxonomy but no support was evident for nearly all the higher level groupings. Nevertheless, some interesting insights were gained in relation to the phylogenetic relationships among some genera under study. Meanwhile, the phylogenetic relationship among Mahseer fishes (genus *Tor* and *Neolissochilus*) were poorly resolved using the current data alone, but the taxonomic revision of other genera particularly for the genus *Puntius* could improve this. The current study suggest that *P. binotatus* and *P. sealei* could be representative of the genus *Puntius*, while any other species identified as belonging to the genus *Puntius* should cluster with this group. The study also revealed that two morphologically similar *Barbonymus* species (namely, *B. gonionotus* and *B. schwanenfeldii*) were phylogenetically distinct (13.0% K2P genetic distance). This indicated that a taxonomic revision of *B. gonionotus* would be required from its current position within the genus *Barbonymus*. The results of the current study also revealed two interesting findings for *Hampala*; (1) the Borneo endemic *Hampala* forms are distinct from the widespread *H. macrolepidota*, and (2) two distinct lineage were evident in *H. bimaculata* from Sarawak. In general, the sequence analysis of the cytochrome *b* mtDNA region has been proven to be useful for assessing phylogenetic relationships among indigenous freshwater fishes in Malaysia.

Keywords: Cytochrome *b*, sequence, mitochondrial DNA, molecular phylogeny

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INTRODUCTION

The Southeast Asian region, which includes Peninsular Malaysia and the island of Borneo, has one of the highest diversity of freshwater fishes in the world (Zakaria Ismail, 1990). According to FishBase, more than 600 species of freshwater fish are recorded in Malaysia (Froese & Pauly, 2004). In Peninsular Malaysia, about 400 freshwater fish species have been described (Mohsin & Ambak, 1983), while more than 350 species of freshwater fishes have been recorded in Borneo (Inger & Chin, 1962; Roberts, 1989; Kottelat *et al*., 1993). The Family Cyprinidae forms the largest family in terms of number of genera and species, and it dominates almost every water body in the region (Mohsin & Ambak, 1983; Zakaria Ismail, 1990). Fishes in the sub-family Cyprininae (i.e. genera *Barbodes*, *Barbonymus*, *Cyclocheilichthys*, *Hampala, Osteochillus, Puntius* and *Tor*) are the most speciose species in Malaysia (Inger & Chin, 1962; Mohsin & Ambak, 1983).

Indigenous freshwater fishes in Malaysia play an important role not only as the main source of protein for rural populations but also as an important source of livelihood in terms of fish trading (Khan *et al*., 1996, Litis *et al*., 1997). Apart from their importance as a food source, many freshwater fishes (e.g. carps) are also favoured as ornamental fishes (Mohsin & Ambak, 1983; Ng, 2004).

Since indigenous freshwater fish are an important source for the region, a comprehensive study on their systematic relationships, particularly of the cyprinids, will assist appropriate management and conservation. Unfortunately, systematic studies of Malaysian cyprinid fishes are scarce and their taxonomy is poorly understood. Current systematic classifications are based solely on their morphological, physiological and other assayable external phenotypic characteristics (Inger & Chin, 1962; Mohsin & Ambak, 1983; Roberts, 1989; Kottelat *et al*., 1993). Meanwhile, conventional systematic characters can often be unreliable because they can be influenced by environmental and non-genetic factors (Vrijenhoek, 1998). On the other hand, DNA-based characters are unlikely to be influenced by environmental pressures (Briolay *et al*., 1998). Furthermore, they are heritable traits, and confidence can therefore be placed on the amount and nature of the genetic information obtained (Avise, 1994).

The development of appropriate molecular marker has strengthened the genetic, taxonomic and systematic studies of fish (Stepien & Kocher, 1997). Meanwhile, the advent of polymerase chain reaction (PCR) technology has greatly facilitated the examination of genetic variation in natural populations (Amos & Hoelzel, 1992). The combination of PCR, with the availability of "universal primers" (e.g. Kocher *et al*., 1989) has enabled a rapid amplification of specific sequences without the need for cloning procedures. The analysis of DNA sequence polymorphisms can provide the highest resolution of genetic variation in cytoplasmic markers with mitochondrial DNA (mtDNA), now a popular tool for constructing phylogenetic relationships. This marker has been applied to resolve questions in biodiversity, conservation genetics and molecular systematic studies (Amos & Hoelzel, 1992; Avise, 1994; Avise & Hamrick, 1995; Stepien & Kocher, 1997).

The present study therefore attempted to construct a molecular-based phylogeny for a number of freshwater fish taxa in Malaysia, particularly from the Cyprinidae family, using partial DNA sequencing of the Cytochrome *b* (Cyt *b*) mtDNA gene.

MATERIALS AND METHODS

Sample Sources and DNA Extraction

The samples were obtained from various river systems in Sarawak, Sabah and Peninsular Malaysia (Table 1). The full samples were recognized morphologically using keys provided by Inger and Chin (1962), Mohsin and Ambak (1983), and Kottelat *et al*. (1993). A total of twenty-three species of cyprinid fishes, representing twelve genera, were examined in this study. The specimens were collected using

TABLE 1
Scientific and local names of cyprinid fishes and outgroup, sampling location, sample size and GenBank accession numbers used in this study

	Subfamily	Scientific name	Local name	Location			Sample size	GenBank Accession No.
				PM	Swk	Sbh		
Family: Cyprinidae	Cyprininae	*Barbodes collingwoodii*	Kepiat		√	√	2	AY243348, DQ366151
	Cyprininae	*Barbonymus gonionotus*	Lampam jawa			√	2	DQ366152, DQ366153
	Cyprininae	*Barbonymus schwanenfeldii*	Tengadak/Lampam sungai	√	√		2	AY355438, AY355426
	Cyprininae	*Cyclocheilichthys apogon*	Boeng/Cemperas		√		2	AY243347, DQ366154
	Cyprininae	*Hampala bimaculata* Type A	Juak/Barop		√		2	AY697362, AY697375
	Cyprininae	*Hampala bimaculata* Type B	Juak/Barop		√	√	1	AY697383
	Cyprininae	*Hampala* intermediate	Barop			√	2	AY697396, AY697397
	Cyprininae	*Hampala macrolepidota*	Adong/Sebarau	√	√		2	AY697310, AY697345
	Cyprininae	*Hampala sabana*	Barop			√	1	AY697406
	Cyprininae	*Hypsibarbus wetmorei*	Krai	√			1	DQ366155
	Cyprininae	*Lobocheilos bo*	Kulong		√		2	DQ366156, DQ366157
	Cyprininae	*Neolissochilus hexagonalepis*	Kejor/Tengas	√			1	DQ366150
	Cyprininae	*Neolissochilus stracheyi*	Kelah	√			2	DQ366168, DQ366169
	Cyprininae	*Osteochillus hasseltii*	Bantak/Pait/Terbul		√		2	AY243346, DQ366160
	Cyprininae	*Osteochillus spilurus*	Bantak/Pait/Terbul			√	2	DQ366161, DQ366162
	Cyprininae	*Osteochillus sp.*	Bantak		√		2	DQ366158, DQ366159
	Cyprininae	*Puntioplites bulu*	Mengalan/Tengalan		√		2	AY243349, DQ366163
	Cyprininae	*Puntius binotatus*	Sisik tebal/Bangah	√	√		2	AY697411, AY365025
	Cyprininae	*Puntius bramoides*	Kachong/Salap			√	1	DQ366164
	Cyprininae	*Puntius sealei*	Mata merah		√	√	2	DQ366165, DQ366166
	Cyprininae	*Tor douronensis*	Semah/Kelah/Pelian		√	√	2	AY243356, DQ366167
	Cyprininae	*Tor tambroides*	Kelah/Empurau	√			1	DQ366170
	Danioinae	*Leptobarbus hosii*	Sayan		√		1	AY243350
Outgroup (Helostomatidae)		*Helostoma temminckii*	Biawan/Tebakang		√		1	AY697412
				Total			40	

PM= Peninsular Malaysia; Swk= Sarawak; Sbh= Sabah

cast-nets, pole-nets or were electro-fished with whole fish preserved in 95% ethanol. The total DNA was extracted from the muscle tissue using a CTAB method (Grewe *et al*., 1993). The quality and approximate yield of DNA were determined through electrophoresis in a 1% agarose gel containing ethidium bromide run at 90V for 30 minutes and visualized under UV light.

DNA Sequencing

A set of primers were used to partially amplify the Cyt *b* gene; 5'-TGACT TGAAR AACCA YCGTT G- 3' known as GluDG-L (Palumbi *et al*., 1991) and 5'-CCCTC AGAAT GATAT TTGTC CTCA- 3' known as CB2-H. Approximately 50-100ng of DNA template was amplified in a 25µl reaction mixture containing 50mM 10X Buffer, 2mM $MgCl_2$, 0.2 µM each dNTP (Fermentas), 0.1 µM of each primer, and 0.5 units of *Taq* DNA Polymerase (Fermentas). The cycle parameters consisted of 25 cycles of denaturation (95°C, 30 sec), annealing (47°C, 30 sec), and extension (72°C, 60 sec). The PCR products were further purified using DNA purification kits (Fermentas and Promega) according to the manufacturers' instructions. The purified PCR products were directly sequenced using the BigDye® Terminator v3.0 Cycle Sequencing kit (ACGT) on an ABI 377 automated sequencer (PE Applied Biosystem) using only the forward primer (GluDG-L).

Statistical Analysis

Multiple alignments of the sequences were conducted using ClustalX software (version 1.81; Thompson *et al*., 1997), and aligned subsequently by eye. The pairwise genetic distance between each cyprinids was calculated using the Kimura two-parameter evolution model (Kimura, 1980) implemented in MEGA version 4.0 (Tamura *et al*., 2007). Meanwhile, the saturation test for all the codons was done using DAMBE version 5.0.66 (Xia & Xie, 2007), and the phylogenetic relationships were inferred using two methods, namely distance analysis using the neighbour-joining method (NJ) and the unweighted maximum parsimony (MP) analysis using close-neighbour-interchange, CNI option) implemented in MEGA. Phylogenetic trees inferred from the Cyt *b* sequences were rooted with *H. temminckii* (family: Helostomatidae, kissing goramy) as the outgroup. Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replicate data sets.

RESULTS AND DISCUSSION

Forty sequences were obtained from twenty-four species (total length of 408 base pairs) and were used for phylogenetic analyses (two individuals of *Leptobarbus hosii* with slightly shorter sequences of 393 and 396 bp respectively were also typed). From the aligned sequences, 186 sites were variable and 144 were phylogenetically informative. The base compositions of sequences were similar to that of the previously reported fish Cyt *b* sequences (Cantatore *et al*., 1994). Across the cyt *b* sequences, the nucleotide composition among the cyprinid fishes screened showed an anti-G bias, which is the characteristic of this mitochondrial gene (Cantatore *et al*., 1994; Briolay *et al*., 1998). The saturation test done onto the sequences at each codon, specifically the third codon which is known to have a faster rate of transition and the transversion showed that the transition at the third codon position was saturated (*Fig. 1*). The estimated transition : transversion ratio is approximately 1.7:1.

The genetic distances among the species were estimated with the Kimura two-parameter model (Kimura, 1980). Table 2 shows the genetic distances among the twenty-three fish species analyzed. *Neolissochilus hexagonalepis* was closely related to its sister taxa *N. stracheyi* - distance value of 0.5% (Table 2). Table 3 further summarizes the average genetic distances among the fish genera. The lowest genetic distance between the genera was observed between Genus *Neolissochilus* and Genus *Tor* (6.2%), while the highest genetic distance was between the Genus *Hampala* and Genus *Hypsibarbus* (18.8%). All the cyprinid sequences were

TABLE 2
Pairwise distance (%) among twenty-three species of cyprinid fishes analyzed based on the Cyt *b* gene. The distances were calculated using Kimura's two-parameter model of nucleotide substitution

		1	2	3	4	5	6	7	8	9	10	11
1	Barbodes collingwoodii											
2	Barbodes gonionotus	12.0										
3	Barbonymus schwanenfeldii	8.7	12.9									
4	*Cyclocheilichthys apogon*	10.4	11.4	11.9								
5	*Hampala macrolepidota*	17.3	17.2	19.9	19.0							
6	*Hampala bimaculata* Type A	17.2	14.8	16.5	17.1	11.5						
7	*Hampala bimaculata* Type B	14.7	17.2	15.4	15.9	13.3	9.6					
8	*Hampala sabana*	15.7	15.8	16.0	16.4	14.5	8.1	8.2				
9	*Hampala* intermediate	17.1	16.1	16.0	17.5	13.2	6.9	8.2	6.8			
10	Hypsibarbus wetmorei	12.9	13.7	13.4	12.9	20.3	18.6	16.1	18.1	19.1		
11	*Lobocheilos bo*	15.2	15.0	15.6	14.7	20.6	17.6	18.3	16.6	18.0	17.1	
12	Neolissochilus hexagonalepis	12.3	14.5	13.4	13.3	18.9	15.9	12.9	13.4	14.1	17.3	17.2
13	*Neolissochilus stracheyi*	12.3	14.5	13.4	13.3	19.5	16.2	12.8	13.4	14.1	17.3	17.0
14	*Osteochillus* sp.	14.8	17.5	14.3	15.3	19.1	17.4	15.9	15.7	15.7	16.7	16.6
15	*Osteochillus hasseltii*	13.9	15.4	15.8	13.5	19.5	19.5	17.4	15.4	17.7	16.5	16.9
16	*Osteochillus spilurus*	16.3	18.0	16.9	16.8	19.9	17.2	17.0	16.4	17.2	16.4	15.4
17	*Puntioplites bulu*	9.5	10.1	10.0	10.8	19.1	18.1	15.6	17.3	17.3	13.0	15.1
18	*Puntius binotatus*	13.6	17.4	14.3	14.8	15.6	17.0	16.2	17.4	16.4	15.1	17.8
19	*Puntius bramoides*	10.7	14.4	11.7	12.7	17.1	15.1	16.3	16.9	17.2	12.7	15.8
20	*Puntius sealei*	14.1	15.8	15.1	16.9	17.0	14.7	13.9	14.7	15.2	18.1	17.7
21	*Tor douronensis*	12.6	16.0	13.7	14.5	18.7	15.7	15.3	15.2	16.3	17.0	16.8
22	*Tor tambroides*	12.6	12.9	13.7	12.9	16.1	15.2	14.1	14.7	14.8	16.3	17.2
23	*Leptobarbus hosii*	15.1	15.5	15.1	16.8	16.2	18.6	15.6	16.6	15.9	16.8	17.4
24	*Helostoma temminckii*	23.9	25.6	26.1	27.1	28.6	28.3	27.9	26.7	27.2	31.3	29.8

Table 2 (*continued*)

		12	13	14	15	16	17	18	19	20	21	22	23	24
13	*Neolissochilus stracheyi*	0.5												
14	*Osteochillus* sp.	16.1	16.1											
15	*Osteochillus hasseltii*	15.3	15.3	11.1										
16	*Osteochillus spilurus*	16.5	16.5	12.3	12.0									
17	*Puntioplites bulu*	13.3	13.3	12.2	12.4	14.8								
18	*Puntius binotatus*	16.0	16.0	13.5	15.1	13.4	12.3							
19	*Puntius bramoides*	15.2	15.2	15.3	16.5	16.5	13.9	14.9						
20	*Puntius sealei*	15.0	15.7	18.0	16.1	16.4	13.4	12.1	16.0					
21	*Tor douronensis*	6.2	6.5	17.3	15.6	16.1	14.7	17.1	15.0	16.6				
22	*Tor tambroides*	5.4	5.9	16.8	15.9	17.1	13.6	16.7	14.2	16.3	6.2			
23	Leptobarbus hosii	17.8	18.5	15.7	15.8	16.1	14.0	18.1	16.7	17.2	16.8	15.4		
24	*Helostoma temminckii*	25.7	25.5	26.6	26.2	25.1	24.6	26.8	28.4	27.1	26.1	25.7	24.5	

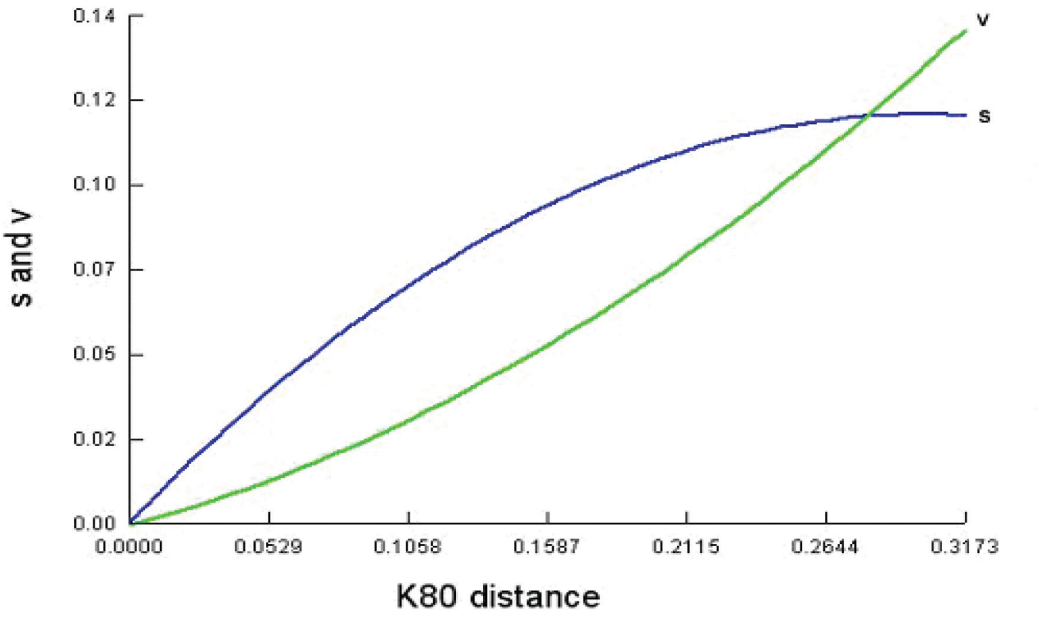

Fig.1: Plot of transition (s) and transversion (v) against divergence using Tamura and Nei (1993) distance method onto the third codon position showing saturation of the transition codon of the Cyt b *gene*

distantly related from the outgroup species, *H. temmincki* (Family Helostomatidae), with the distance values ranging from 23.9% to 31.3%, respectively (Table 3).

Fig. 2 presents the phylogenetic tree recovered from the partial Cyt *b* sequences of thirty-nine cyprinid individuals and one outgroup species, constructed using both NJ and MP methods (only the NJ tree is presented since tree topologies are very similar). Although the phylogeny appeared to match the major groupings currently recognized in the taxonomy, no support was evident for nearly all higher level groupings. Thus, it is clear that further work is needed to clarify the relationships between the many genera.

The phylogenetic analysis grouped the two genera of Mahseer fishes that exist in Malaysia, namely, Genus *Neolissochilus* and Genus *Tor*. The relationship between the two species in the genus *Tor* (*T. douronensis* and *T. tambroides*) was poorly resolved using the NJ and MP methods. In contrast, a close relationship between *N. hexagonalepis* with *N. stracheyi* is supported by a strong bootstrap value (>99%). Nonetheless, a more variable mtDNA marker (e.g. control region or COI genes) or longer sequence of mtDNA genes may be required to further resolve systematic relationship among Mahseer species and populations.

A recent revision on the taxonomic classification of fishes within the genus *Puntius* has shown that some previously recognized taxa have been assigned to new genera: *Puntius collingwoodii* (Kottelat *et al*., 1993) to *Barbodes collingwoodii* (Martin-Smith, 1996), *Puntius javanicus* (Davidson, 1975) to *Barbonymus gonionotus* (Kottelat, 2001), *P. schwanenfeldii* (Vidthayanon *et al*., 1997) to *Barbonymus schwanenfeldii* (Kottelat, 2001), *P. daruphani* (Vidthayanon *et al*., 1997) to *Hypsibarbus wetmorei* (Kottelat, 2001) *and P. bulu* (Kottelat *et al*., 1993) to *Puntioplites bulu* (Kottelat & Whitten, 1996). The phylogenetic analysis using both NJ and MP has shown that all previously described species in the genus *Puntius* are

TABLE 3
A summarized of average pairwise genetic distance (%) among the cyprinids fishes and the outgroup (*Helostoma*) of the Cyt *b* gene. The distances were calculated using Kimura's two-parameter model of nucleotide substitution

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Barbodes*													
2	*Barbonymus*	10.4												
3	*Cyclocheilichthys*	10.4	11.6											
4	*Hampala*	16.7	16.6	17.4										
5	Hypsibarbus	12.9	13.5	12.9	18.8									
6	*Lobocheilos*	15.2	15.3	14.7	18.4	17.1								
7	Neolissochilus	12.3	13.9	13.3	15.7	17.3	17.0							
8	*Osteochillus*	15.0	16.3	15.2	17.7	16.5	16.3	15.9						
9	*Puntioplites*	9.5	10.0	10.8	17.7	13.0	15.1	13.3	13.1					
10	*Puntius*	13.2	15.1	15.2	16.0	15.8	17.4	15.6	15.6	13.1				
11	*Tor*	12.6	14.3	14.0	16.0	16.8	16.9	6.2	16.4	14.4	16.4			
12	*Leptobarbus*	15.1	15.3	16.8	16.7	16.8	17.4	18.3	15.8	14.0	17.5	16.3		
13	*Helostoma*	23.9	25.9	27.1	27.8	31.3	29.8	25.6	26.0	24.6	27.2	26.0	24.5	

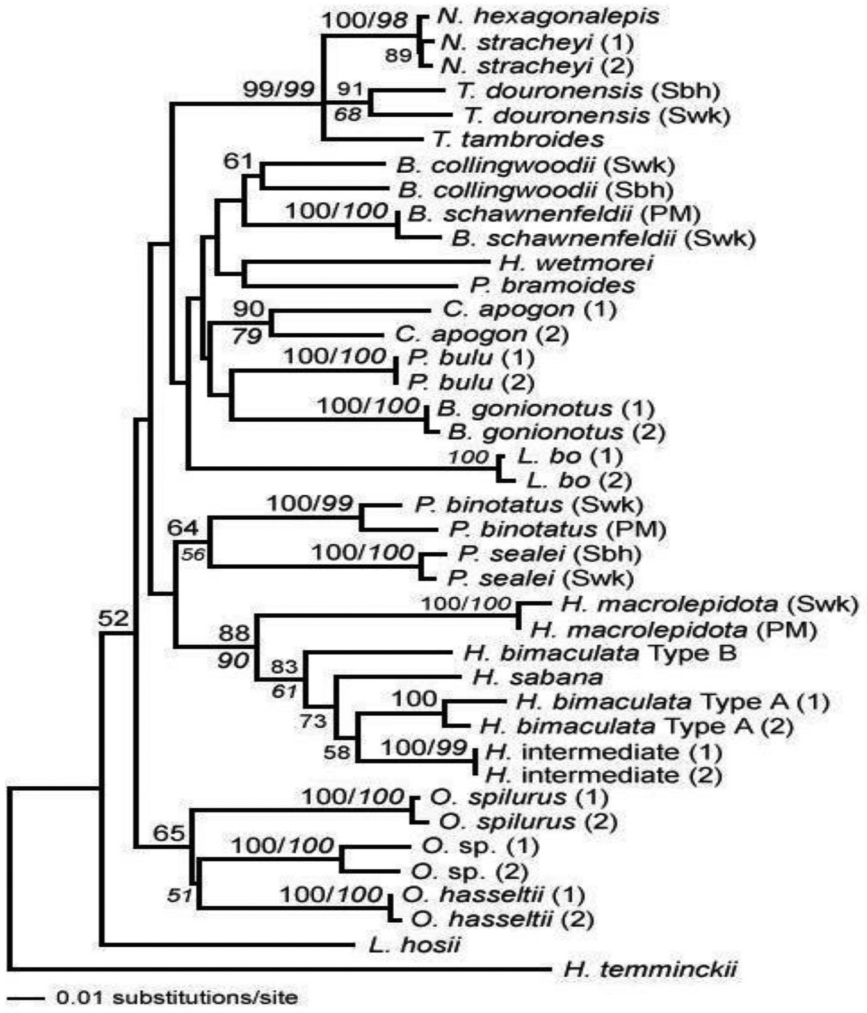

Fig. 2: Phylogenetic relationships of indigenous fishes under study based on the Cyt b *gene of the mtDNA. The values on the branches represent both NJ and MP bootstrap estimates (italic values represents MP analysis), based on 1000 replicates. Only the bootstrap values >50% are shown (PM=Peninsular Malaysia; Swk=Sarawak; Sbh=Sabah)*

divided into two sub-groups; the newly elevated *Puntius* species clustered randomly across the trees, while the remaining two *Puntius* fishes (*P. binotatus and P. sealei*) formed a distinct *Puntius* cluster with strong bootstrap support (>99%). Thus, the current molecular data suggest that *P. binotatus and P. sealei* could be the representative of the genus *Puntius*, and any other species identified as belonging to the genus *Puntius* should cluster with this group.

In addition, the phylogenetic analysis revealed that the two morphologically similar *Barbonymus* species, *B. gonionotus* and *B. schwanenfeldii* did not cluster in a single *Barbonymus* clade. Instead, *B. gonionotus* clustered with *P. bulu* and *Cyclocheilichthys*

apogon while *B. schwanenfeldii* formed a second clade with *B. collingwoodii*, *H. wetmorei* and *P. bramoides*. This result suggests that the morphological similarity between *B. gonionotus* and *B. schwanenfeldii* may result from the convergent evolution rather than co-ancestry. *B. gonionotus* is not native to Peninsular Malaysia, as it was introduced from Java at the beginning of the 19th century (Welcomme, 1981). In Malaysia, this particular exotic species has since bred well in ponds and in natural river systems where it was introduced. Nowadays, *B. gonionotus* is found living in sympatry with *B. schwanenfeldii* in many river systems. Nonetheless, some recent molecular studies using Cyt *b* mtDNA RFLP fragment analysis (Esa & Khairul, 2003) of the two species from the sites where they are sympatric in the Serting River (Negeri Sembilan) did not find any evidence for hybrid introgression, supporting their genetic distinctiveness (distance value of 13.0%).

The genus *Hampala* was one of the main focuses of the current study. As indicated earlier on, the phylogenetic analysis produced slightly different NJ and MP topologies, particularly in relation to the relationships among *H. bimaculata*, *H. sabana* and an undescribed *Hampala* taxa (known in this study as the intermediate form). Two important findings were investigated further within the genus *Hampala*. First, the widespread *H. macrolepidota* was phylogenetically distinct from other Borneo endemic *Hampala* taxa and was an older lineage than the other forms. Secondly, this study identified two monophyletic *H. bimaculata* haplotypes, with different geographical distributions (Type A from Southern and Central Sarawak, Type B from Northern Sarawak and the West Coast of Sabah). In other words, they were found to represent distinct mtDNA lineages. Therefore, a thorough and more detailed molecular study on the phylogeography and phylogenetic relationships among *Hampala* fishes should provide better insights into the systematic (and taxonomic) status and evolutionary history of this interesting genus.

Overall, the taxonomy and systematic of freshwater fishes in Malaysia are fragmented and poorly resolved. The current study has provided a robust attempt to reconstruct the phylogeny of a number of cyprinid taxa in Malaysia using the molecular approach. Indeed, the molecular data generated here have shown that a molecular approach could be very useful in clarifying the systematic status of the Malaysian freshwater fish species. In addition, the richness and high biodiversity of the fauna particularly freshwater fishes should be properly documented for appropriate management and conservation. Meanwhile, more molecular studies should be undertaken to examine larger datasets of cyprinids and related families in order to obtain a more comprehensive understanding of their systematic relationships.

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Design of an Object-oriented Framework for Modelling the Partitioning of Captured Solar Radiation and Evapotranspiration in Intercropping Systems

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ABSTRACT

x-library is a C++ object-oriented framework for modelling the partitioning of captured solar radiation and evapotranspiration in intercropping systems. The design and analysis of the x-library are done to ensure that the soil-plant-atmosphere system is categorised into classes, such as weather, microclimate, intercrop, crop, canopy, leaf, roots, soil, heat, and radiation. Meanwhile, x-library implements two kinds of solar radiation models; namely, one-dimensional (1-D), and two-dimensional (2-D) model, where irradiance varies in one dimension (vertical) and in two dimensions (vertical and horizontal), respectively. Radiation partitioning is based on weighting criteria so that a crop having the larger leaf area index and extinction coefficient would have greater share of captured radiation. Evapotranspiration partitioning is calculated using the Shuttleworth-Wallace equation. Model comparisons with a field experiment showed an overall good agreement between the simulated and measured solar radiation and transpiration values. A graphical user interface front-end for the x-library known as the x-model was also developed, primarily for non-modellers and non-programmers.

Keywords: Intercrop, model, object-oriented, reusability

INTRODUCTION

When two or more crops are grown together in an intercropping system, these crops may succeed in higher yields or they may fail. In order to understand the outcome of such crop combinations, it is necessary to be able to quantify the processes involved in the partitioning of resources in this particular system. This is because these resources, such as solar radiation and water, will determine not only the growth and development of individual crop species, but also the community as a whole (Wallace, 1997).

Consequently, capturing of solar radiation and evapotranspiration must be modelled together. It is important to note that plants rarely compete for solar radiation without simultaneously competing for water as well (Cannel & Grace, 1993; Wallace, 1995). The first modelling step is to quantify the partitioning of the captured radiation in mixed canopies, and this is followd by using this information in the evapotranspiration model to drive the processes

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of evaporation and transpiration which are strongly radiation-dependent (Wallace, 1997).

Although several such coupled radiation-evapotranspiration models exist (e.g., Kropff, 1993), most of them are implemented using procedural or structured programming (e.g., FORTRAN and BASIC), rather than using object-oriented programming (OOP). The primary advantage of having an object-oriented modelling framework is to achieve model reusability and extendibility, which are two highly desirable properties in model sharing and development. These two key properties facilitate: (i) the interchanging of component models within and between the whole-system models, (ii) incremental model development without having to rewrite existing code, and (iii) maintenance of more than one model of a component (van Evert and Campbell, 1994). Recognising these benefits, agricultural workers are using OOP more frequently in their modelling work (e.g. Pan *et al*., 2000; Salminen *et al*., 2005; Aumann, 2007; Martinez *et al*., 2008; Gocic & Trajkovic, 2010). The scenario was totally different a decade ago, when the production of high quality software was rarely a high priority in modelling work (McCown *et al*., 1996). This was because models were commonly built to be accurate and to meet immediate research objectives, but they were rarely built to be reusable or extendible. Moreover, models were also usually built and used in limited situations, and then discarded for newer ones. This has seriously hampered both the progress and the use of the existing models. Loomis (1985) and Seligman (1990) emphasise that modelling progress is best achieved by testing and improving the best of the existing models rather than writing new ones from scratch, which is akin to trying to "reinvent the wheel".

Consequently, the objective of this paper is to introduce a design of an object-oriented framework called x-library specifically for modelling the partitioning of captured radiation and evapotranspiration in intercropping systems. x-library has been designed following the principles of the object-oriented software design as specified by Meyer (1997) so that it would be reusable and extendible to achieve ease of model sharing and further development. While x-library is intended for modellers and programmers, x-model has also been developed as a graphical user interface front-end for x-library that is intended for non-modellers and non-programmers.

OBJECT-ORIENTED DESIGN AND ANALYSIS OF THE X-LIBRARY

x-library is written in C++ and conforms to the C++ standards set by the ISO (International Organization of Standardization). Thus, x-library is independent of hardware and type of operating system. x-library was primarily compiled and tested using Microsoft Visual C++ 2003.

Nonetheless, x-library can only be used as part of the developer's project. This means that the x-library cannot be compiled into an executable file by itself and then run. Developers must create a project, along with the x-library, to develop an executable program specific to the developer's intended hardware and operating system. There are two ways how x-library can be used in a project. The first is to compile all the x-library source files to produce a single library file. This library file is then linked with the developer's project. The second method is to include only the necessary x-library source and header files in the project, which are then compiled and linked together with the developer's own source files. The x-library can later be used to create a GUI (Graphical User Interface) Windows or Mac program, a command line driven DOS program, or a UNIX program.

The design and analysis of the x-library are done such a way that soil-plant-atmosphere system is categorised into several components (or classes), such as weather, microclimate, intercrop, crop, canopy, leaf, roots, soil, heat, and radiation (Table 1 and *Fig. 1*). The weather classes deal with meteorological conditions such as the daily and hourly weather properties, incoming solar radiation, and solar position. Meanwhile, the heat and solar radiation

TABLE 1
x-library classes

Group	Sub-group	Classes
Intercrop		XIntercrop, XICell
Crop		XCrop, XCrpLayer, XCrpCell
Canopy		XCanopy, XCnpyLayer, XCnpyCell
Leaf		XLeaf, XLeafCell, XLeafLin, XLeafPoly, XGrpLeaf
Root		XRootSys
Microclimate		XMicroclimate
Weather		XAstro, XDayWthr, XDiuWthr
Radiation		XRad, XRadComp, XRadLayer, XRadCell
Heat		XHeat, XSrc, XEvpSW
Soil		XMoistDist, XSoil, XSoilBasic, XSoilEnv
File		XFile
Support	Base	XObject
	Date	XDate
	Distributional	XDist, XCurveFit
	Directional	XDir
	Factory	XFactory, XFactoryLayer, XFactoryCell
	Exception	Exception, GeneralError, SpecificError, AccessEmpty, AccessNullPtr, WrongClass, OutOfRange, RangeTooSmall, AbnormalError, BadArg, BadState, DivideByZero, InitFail, NoLeaf

classes compute the evapotranspiration and the partitioning of captured radiation, respectively. A class that unifies the various components of the soil-plant-atmosphere system is the microclimate class, XMicroclimate. This microclimate class contains pointers to the heat base class (XHeat), radiation base class (XRad), weather base class (XAstro), soil base class (XSoil), and the intercrop base class (XIntercrop), in which the microclimate class acts as a link or common channel in which the various soil-plant-atmosphere classes can be used to call or use each other to obtain or share information. For example, the calculation of the crop roughness length and zero plane displacement is done in the heat classes (XHeat, XSrc and XEvpSW). However, these calculations require information such as crop height and leaf area index, which are obtained via the microclimate class from the crop classes (XCrop, XCrpLayer and XCrpCell).

The intercrop class is implemented to describe the cropping system as comprising one or more crop species, whereby each crop is in turn described as consisting of roots and canopy, where the latter comprises leaves. To implement this particular design, the intercrop base class XIntercrop has a container of crop objects; thus, if a cropping system has two crop species, for example, the container in XIntercrop will then have two crop objects. The crop base class XCrop, on the other hand, has a pointer to the canopy base class XCanopy, and another pointer to the root base class XRootSys. The canopy class, in turn, has a container of leaf objects. Each leaf class (XLeaf, XLeafCell, XLeafLin and XLeafPoly) represents the properties of a single leaf; however, the leaf class XGrpLeaf is unique because it represents the properties of a collection or group of leaves, rather than a single leaf in the canopy. Currently, x-library does not

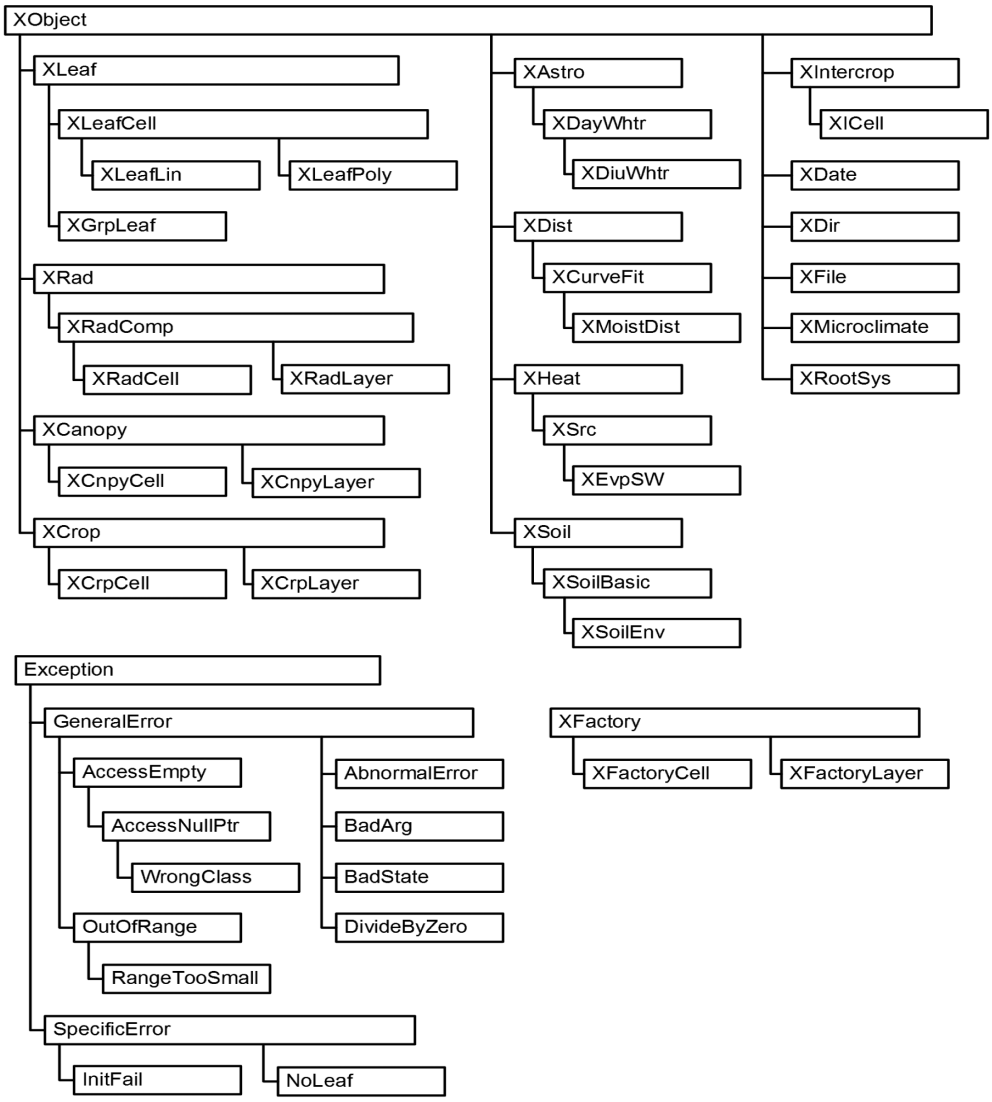

Fig. 1: Class hierarchy for the x-library

have a plant growth model; consequently, users must supply inputs, such as leaf area index (LAI), plant height, as well as the distributions of leaf azimuth and inclination density, for the canopy architecture. Nevertheless, users can implement their own plant growth model and incorporate it into x-library without having to modify the original code to achieve model compatibility because x-library is object-oriented and designed to be reusable and extendible.

The main x-library classes are derived from the abstract base class XObject. This particular base class is not useful by itself, but serves to support polymorphism and provide several common and basic services, such as object persistence, cloning and copying, to its derived classes.

TABLE 2
Classes used specifically by the (a) 1-D radiation model, and (b) 2-D radiation model

a) 1-D Radiation Model

Group	Sub-group	Classes
Intercrop		XIntercrop
Crop		XCrpLayer
Canopy		XCnpyLayer
Leaf		XGrpLeaf
Radiation		XRadLayer
Support	Factory	XFactoryLayer

b) 2-D Radiation Model

Group	Sub-group	Classes
Intercrop		XICell
Crop		XCrpCell
Canopy		XCnpyCell
Leaf		XLeafCell, XLeafLin, XLeafPoly
Radiation		XRadCell
Support	Factory	XFactoryCell

Weather

The weather classes handle daily and diurnal meteorological properties, which include solar radiation, air temperature, vapour pressure and wind speed. These weather data are usually stored in a pre-defined format in text files. Given the day and hour of simulation, as well as the latitude of the sites, these classes can calculate certain properties such as the solar position and day length.

Certain meteorological properties (such as solar position, solar declination, day length, time of sunrise and sunset, as well as total daily solar irradiance and its partitioning into direct and diffuse solar radiation components) are calculated according to the equations from Spitters (1986), Kropff (1993), and Campbell and Norman (1998).

Plant-radiation Regime

The x-library implements two kinds of plant-radiation regime models [a one-dimensional (1-D), and a two-dimensional (2-D) model], where the former models the plant-radiation regime in one dimension and irradiance varies only vertically, whereas the latter models the plant-radiation regime in two dimensions, where irradiance varies both vertically and horizontally. The 1-D and 2-D solar radiation models were specifically implemented in the XRadLayer and XRadCell radiation classes, respectively. However, both of these classes are supported by a specific set of other classes, as shown in Table 2. To perform its calculations, XRadLayer requires information from the intercrop base class (XIntercrop), as well as from the derived classes of crop (XCrpLayer), canopy (XCnpyLayer), and leaf (XGrpLeaf). Likewise, the radiation calculations in the XRadCell class

require information that is only available from the derived classes of the intercrop (XICell), crop (XCrpCell), canopy (XCnpyCell), and leaf (XLeafCell, XLeafLin and XLeafPoly).

One-dimensional (1-D) Modelling

In the 1-D radiation modelling, the canopy architecture or foliage distribution must first be known. Information regarding canopy architecture is calculated in the crop, canopy and leaf classes, and passed on to the XRadLayer radiation class. The foliage distribution of a crop is characterised mathematically using the G-function defined by Ross and Nilson (1965), as:

$$G(\theta,\varphi) = \frac{1}{2\pi}\int_0^{2\pi}\int_0^{\pi/2} g(\theta_L,\varphi_L)\,|\cos r_L r|\,\sin\theta_L\, d\theta_L\, d\varphi_L \quad [1]$$

where cos $r_L r$ is the cosine angle between the leaf normal direction r_L and sun direction r, and is calculated by:

$$\cos r_L r = \cos\theta\cos\theta_L + \sin\theta\sin\theta_L\cos(\varphi-\varphi_L) \quad [2]$$

where (θ, ϕ) is the solar position (inclination and azimuth, respectively), and (θ_L, ϕ_L) is the leaf position (inclination and azimuth, respectively). Equation (1) is numerically integrated using the equation given by Lemeur (1973a, b):

$$G(\theta,\varphi) \approx \sum_{j=1}^{12}\left\{\sum_{i=1}^{16}\left[G'(\varphi_L)\right]_{\frac{\pi}{8}(i-1)}^{\frac{\pi}{8}i} \cdot\left[G'(\theta_L)\right]_{\frac{\pi}{24}(j-1)}^{\frac{\pi}{24}j}\cdot\left[|\cos r_L r|\right]_{\frac{\pi}{24}(j-1),\frac{\pi}{8}(i-1)}^{\frac{\pi}{24}j,\frac{\pi}{8}i}\right\} \quad [3]$$

where π/2 (total inclination range) is divided into 12 equal successive parts, and 2π (total azimuth range) into 16 parts; G'(θ_L) is the cumulative distribution function of the leaf normal inclination, and G'(ϕ_L) is the cumulative distribution function of the leaf normal azimuth.

G(θ, ϕ) is corrected to account for the radiation scattering by leaves and when the leaves are not randomly distributed, but strongly clumped along planting rows, it is thus:

$$\hat{G}(\theta,\varphi) = G(\theta,\varphi)\cdot\sqrt{1-\sigma}\cdot\Omega(\theta) \quad [4]$$

where $\hat{G}(\theta,\varphi)$ is the corrected G-function; σ is the leaf scattering coefficient of radiation, and Ω(θ) is the clumping factor (Tournebize & Sinoquet, 1995; Campbell & Norman, 1998) which is determined empirically by:

$$\Omega(\theta) = \frac{\Omega_0}{\Omega_0 + [1-\Omega_0]\exp[-2.2\theta^{3.8-0.46\varepsilon}]} \quad [5]$$

where

$$\Omega_0 = \frac{\ln\left[f_c\exp\left(-k\frac{L}{f_c}\right)+(1-f_c)\right]}{-kL} \quad [6]$$

and ε is the ratio of plant height to width; and f_c is the fractional canopy cover which is the fraction per unit ground area occupied by canopy cover and is approximated by taking the ratio of canopy width to row spacing (Campbell & Norman, 1998). Finally, extinction coefficient k is related to the G-function by:

$$k = \frac{G(r)}{\cos\theta} \quad [7]$$

(Ross & Nilson, 1965; Lemeur, 1973a; Goudriaan, 1988).

Information on the G-function, leaf area index, extinction coefficient, leaf scattering coefficient for radiation, and clump factor is passed to the XRadLayer class. In the XRadLayer class, direct radiation within the mixed canopies with the n number of crops is calculated by:

$$I_{dr} = (1-\rho)I_{0,dr}\exp\left(-\sum_{j=1}^{n}\frac{\hat{G}(\theta,\varphi)_j}{\cos\theta}L_j\right) \quad [8]$$

where $I_{0,dr}$ is the amount of direct radiation above canopy; L_j is the leaf area index of the crop species j; and p is the mean canopy reflection coefficient calculated by:

$$\rho = \frac{(1-\sqrt{1-\bar{\sigma}})}{(1+\sqrt{1-\bar{\sigma}})}\times\frac{2}{(1+1.6\cos\theta)} \quad [9]$$

where $\bar{\sigma}$ is the mean leaf scattering coefficient of radiation for all crops (Goudriaan, 1977, 1988). Diffuse radiation within the mixed canopies is calculated by:

$$I_{df} = (1-p)\int_0^{2\pi}\int_0^{\pi/2} B(\theta,\varphi)\,\exp\left[-\sum_{j=1}^{n}\frac{\hat{G}(\theta,\varphi)_j}{\cos\theta}L_j\right]\cos\theta\,\sin\theta\,d\theta\,d\varphi \quad [10]$$

and integrated numerically using the 5-point Gaussian method (Mathews, 1987; Goudriaan, 1988).

The total amount of direct radiation A_{dr} and the diffuse radiation A_{df} intercepted by all crops are calculated by:

$$A_{dr} = I_{0,dr} - I_{dr}$$
$$A_{df} = I_{0,df} - I_{df} \quad [11]$$

The amount of radiation captured by crop species i is then calculated by:

$$A_{dr,c,i} = A_{dr}\cdot\omega_i$$
$$A_{df,c,i} = A_{df}\cdot\omega_i \quad [12]$$

where $A_{dr,c,i}$ and $A_{df,c,i}$ are the amount of direct and diffuse radiation captured by the crop species i, respectively, and ω_i is determined by:

$$\omega_i = \frac{k_i\,L_i\cdot\sqrt{1-\sigma_i}}{\sum_{j=1}^{n}\left[k_j\,L_j\cdot\sqrt{1-\sigma_j}\;\right]} \quad [13]$$

where k_i and σ_i are the leaf extinction coefficient and the leaf scattering coefficient of radiation, respectively, for crops species i (Tournebize & Sinoquet, 1995).

Two-dimensional (2-D) Modelling

XRadCell radiation class implements the radiation modelling in two dimensions according to the method by Sinoquet and Bonhomme (1992). However, XRadCell collaborates closely its support classes: XICell intercrop class, XCrpCell crop class, XCnpyCell canopy class, and the leaf classes XLeafLin and XLeafPoly. The XICell intercrop class will divide the canopy space into a set of contiguous rectangular cells, forming a two-dimensional grid network, perpendicular to the planting row direction (*Fig. 2*). The aerial space from the soil surface to the canopy top is divided into N_z horizontal layers of thickness E_z, and N_x vertical sections of thickness E_x, where only E_z and E_x are specified by users. N_x and N_z are then calculated by XICell depending on the size of the canopy space, and the given attributes E_z and E_x.

2-D radiation modelling is detailed and complex because the properties of individual leaves must be known (such as the leaf position, leaf inclination and azimuth, and leaf arch or curvature). The properties of each leaf in the crop canopy are consequently represented by the leaf classes XLeafLin and XLeafPoly. The difference between these two leaf classes is that XLeafPoly is for leaves that arch according a second-degree polynomial curve (such as the maize leaves in *Fig. 2*), and XLeafLin is for the leaves that incline at a constant angle (such as the sunflower leaves in *Fig. 2*; this leaf arch type is more common).

In the XRadCell class, the probability $\underline{P_k}$ of the total radiation intercepted within the k-th cell visited by a single beam is calculated by:

$$P_k = \left[\prod_{c=1}^{k-1}\exp\left(-\sum_{j=1}^{n}G_{jc}(r)\cdot\rho_{f,jc}\cdot s_c\cdot\sqrt{1-\sigma_j}\right)\right]\cdot\left[1-\exp\left(-\sum_{j=1}^{n}G_{jk}(r)\cdot\rho_{f,jk}\cdot s_k\cdot\sqrt{1-\sigma_j}\right)\right] \quad [14]$$

where the multiplicative series c=1 to ($\underline{k}$-1) represents every cell visited sequentially by the beam in reaching the target cell k; $G_{jc}(r)$ is the G-function for the $\underline{j}$-th crop in the c-th cell; $\rho_{f,jc}$ is the leaf area density for the j-th crop in the c-th cell; s_c is the beam path length in the c-th cell; $\sigma_{\underline{j}}$ is the leaf scattering coefficient for crop species j; and n is the total number of crops (Tournebize & Sinoquet, 1995). Consequently, the fraction of the total radiation captured by crop species i in the k-th cell F_{ki} is determined by:

$$F_{ki} = P_k\cdot\omega_{ki} \quad [15]$$

where ω_{ki} is determined, similar to Equation (13), by:

$$\omega_{ki} = \frac{k_{ki}\,L_{ki}\cdot\sqrt{1-\sigma_i}}{\sum_{j=1}^{n}\left[k_{kj}\,L_{kj}\cdot\sqrt{1-\sigma_i}\right]} \quad [16]$$

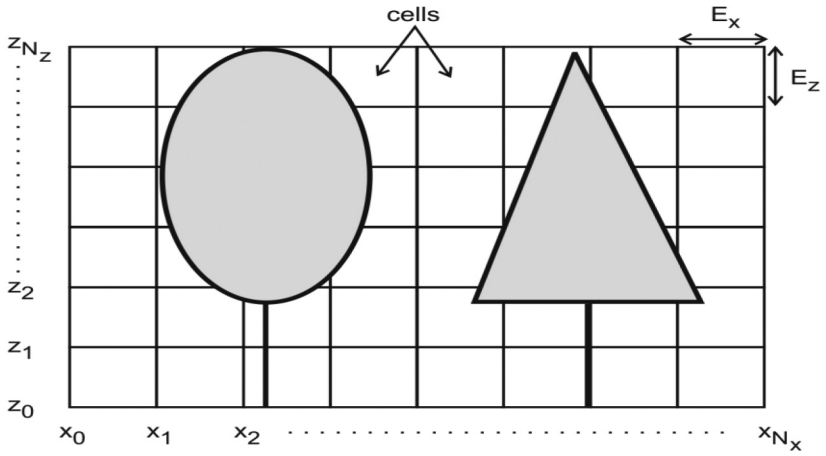

Fig. 2: The 2-D radiation model. The canopy space of an intercropping system is divided into a network of cells, perpendicular to the planting row direction.

where k_{ki} and L_{ki} are the extinction coefficient and the leaf area index of the crop species i in k-th cell, respectively.

Meanwhile, Eq. (14) shows that three properties must be calculated in each cell in the grid network: 1) the G-function, 2) the leaf area density, and 3) the distance travelled within the cell by a single beam. The plant profile method adapted from Stewart and Dwyer (1993) was used to mathematically project a three-dimensional plant architecture to a representative two-dimensional plane, perpendicular to the planting row direction. The plant profile method is implemented in the XCrpCell crop class and XCnpyCell canopy class. If a crop has large or long leaves (like sunflower or maize), a leaf may not lie entirely within a cell; instead, several cells may encompass a single leaf. Thus, XCrpCell and XCnpyCell classes are also to determine the leaf portion or section that is encompassed by a given cell. This is done so to ensure that the calculations on the G-function and leaf area density in a given cell are only based on that encompassed leaf section.

The XRadCell class calculates the distance travelled within a cell by a single beam based on the method discussed in Allen (1974), Gijzen and Goudriaan (1989), and Sinoquet and Bonhomme (1992). In *Fig. 3*, for example, a beam enters the cell at A (x_0, z_0, y_0) described by elevation angle $\underline{\beta}$ to horizontal and at $\underline{\alpha}$ angle from the planting row, and the beam reaches point C at the xz-plane, or (x, z, 0). Thus, it follows that:

$$\frac{\sin\beta}{\cos\beta\sin\alpha} = \frac{CD}{BD} = \frac{z - z_0}{x - x_0} \qquad [17]$$

(Sinoquet and Bonhomme, 1992). The 2-D model is able to track the course of the beam travel within the canopy space because the beam travel must satisfy Equation (17). The beam path length within a cell $\underline{s_c}$ is computed by calculating the intersections between the beam path and cell boundaries, i.e. by making x = vertical cell boundary, or z = horizontal cell boundary, and then computing the path length s_c in the c-th cell (Sinoquet & Bonhomme, 1992).

Note that a beam with the same inclination and azimuth angle can enter at any point on the cell. Thus, the limit on the number of beams to be "pushed" into each cell can be determined by several pre-trial runs to obtain the minimum number of beams that can be used without sacrificing accuracy. Each computed s_c is then substituted into Equation (14) to determine P_k so that the mean probability of the intercepted radiation for beams coming from (β, α) can finally be calculated ($\bar{P}_k$). Meanwhile, direct radiation intercepted by all the crops within cell k is calculated as:

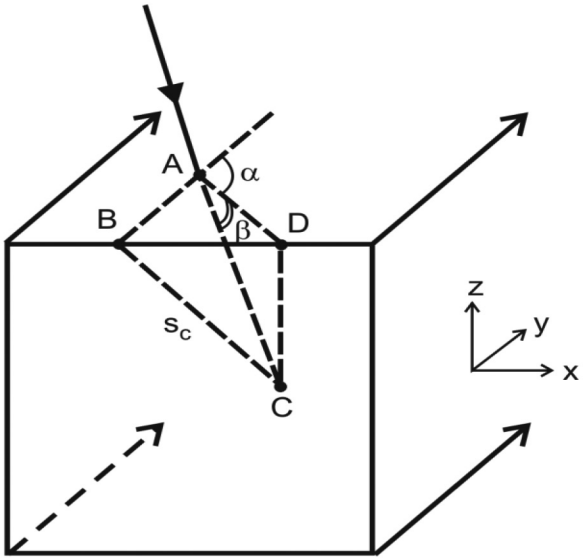

Fig. 3: Coordinate system to track the beam travel course within a cell

$$A_{dr,k} = (1-p)\cdot I_{0,dr}\cdot \bar{P}_k \quad [18]$$

The diffuse radiation intercepted by all the crops within cell k is calculated as:

$$A_{df,k} = (1-p)\sum_{\Omega=1}^{n} I_{0,df(\Omega)}\cdot \bar{P}_{k(\Omega)} \quad [19]$$

where $I_{0,df(\Omega)}$ is the incident diffuse radiation coming from direction Ω under a UOC or SOC sky. To determine $I_{0,df(\Omega)}$, the sky is divided into x equal number of inclination intervals and y equal number azimuth intervals.

Evapotranspiration

The heat class XEvpSW, derived from XSrc and XHeat, determines various heat fluxes in the intercropping system according to the SW equation extended by Wallace (1997). XEvpSW calls the weather, radiation, intercrop and soil classes to retrieve the required information for the various heat flux calculations in the system. In the XEvpSW class, the total latent heat flux of the system with n crops is given by:

$$\lambda E = \sum_{i=1}^{n} C_{c,i} PM_{c,i} + C_s PM_s \quad [20]$$

where

$$PM_{c,i} = \frac{\Delta A + \{\rho c_p D - \Delta r_a^{c,i}(A - A_{c,i})\}/(r_a^a + r_a^{c,i})}{\Delta + \gamma\{1 + r_s^{c,i}/(r_a^a + r_a^{c,i})\}}$$

$$PM_s = \frac{\Delta A + \{\rho c_p D - \Delta r_a^s(A - A_s)\}/(r_a^a + r_a^s)}{\Delta + \gamma\{1 + r_s^s/(r_a^a + r_a^s)\}} \quad [21]$$

$$C_{c,i} = \left(1 + \frac{1/R_s + \sum_{j=1, j\neq i}^{n} 1/R_{c,j}}{\frac{1}{R_{c,i}} + \frac{1}{R_a}}\right)^{-1}$$

$$C_s = \{1 + R_s R_a / R_c (R_s + R_a)\}^{-1} \quad [22]$$

$$R_{c,i} = (\Delta + \gamma)\, r_a^{c,i} + \gamma\, r_s^{c,i}$$

$$R_a = (\Delta + \gamma) r_a^a \quad [23]$$

$$R_s = (\Delta + \gamma)\, r_a^s + \gamma\, r_s^s$$

where c_p is the specific heat of water at constant pressure (4182 J kg^{-1} K^{-1}); D is the vapour pressure deficit, or ; $e_s(T_r) - e_r$ Δ is the mean rate of change of saturated vapour pressure with temperature, or $[e_s(T_r) - e_s(T_0)]/(T_r - T_0)$; γ is the psychometric constant (0.658 mb K^{-1}); A and A_s are the total energy available to the system

and soil, respectively, and $A_{c,i}$ is the amount of energy available to crop i, so that:

$$A_{c,i} = F_i R_n \quad [24]$$

where F_i is the fraction of radiation intercepted by crop species i (obtained from the radiation classes). Thus, F_i can be regarded as the link between radiation and evapotranspiration models. The energy available to the soil A_s is:

$$A_s = (R_n - G) \cdot \left(1 - \sum_{i=1}^{n} F_i\right) \quad [25]$$

And the heat flux into the soil G is calculated by:

$$G = 0.35 \cos\theta \cdot R_n^s \quad [26]$$

(Kustas & Norman, 1999a, 1999b).

The partitioning of the various latent heat fluxes is determined from the total latent heat flux λE which is the sum of all latent heat fluxes in the intercropping system, or in a two-crop intercropping system:

$$\lambda E = \lambda E_s + \lambda E_{c,1} + \lambda E_{c,2}$$
$$= \frac{\Delta A_s + (\rho c_p D_0)/r_a^s}{\Delta + \gamma(1 + r_s^s/r_a^s)} + \frac{\Delta A_{c,1} + (\rho c_p D_0)/r_a^{c,1}}{\Delta + \gamma(1 + r_s^{c,1}/r_a^{c,1})} + \frac{\Delta A_{c,2} + (\rho c_p D_0)/r_a^{c,2}}{\Delta + \gamma(1 + r_s^{c,2}/r_a^{c,2})} \quad [27]$$

where D_0 is the vapour pressure deficit at the canopy source height, or

$$D_0 = D + \frac{r_a^a}{\rho c_p}\{\Delta A - (\Delta + \gamma)\lambda E\} \quad [28]$$

The SW model required several resistance components to be known. These include r^a_a (resistance between mean canopy flow and reference height); $r^{c,i}_s$ (bulk stomatal resistance); $r^{c,i}_a$ (bulk canopy boundary layer resistance); r^s_a (resistance between soil and mean canopy flow); and r^s_s (soil surface resistance). All components of these resistances are calculated in the heat classes, and they obtain information about the weather, crop and soil properties by calling the weather, crop and soil classes, respectively.

The aerodynamic resistance between the soil surface and the sink for momentum in the vegetation r^s_a is given by Shuttleworth and Gurney (1990), and Shuttleworth (1991) as:

$$r_a^s = \frac{h \cdot \exp(\eta)}{nK(h)} \cdot \left\{\exp\left[-\eta \cdot z_{s0}/h\right] \quad \text{e} \quad \eta \right.$$
$$\left. - \exp\left[-\eta \cdot z_0 + d/h\right]\right\} \quad [29]$$

where h is the crop height; η is the attenuation coefficient for eddy diffusivity taken as 3.0, which is typical for most agricultural crops (Monteith, 1975); z_{s0} is the roughness length soil surface taken as 0.004 m for bare, tilled soil surface (Hansen, 1993); z_0 and d are the crop roughness length and zero displacement height, respectively; and K(h) is the eddy diffusivity at crop height h, and is calculated by:

$$K(h) = k^2(h - d)u(z_x)/\ln\{(z_x - d)/z_0\} \quad [30]$$

where z_x is the reference height; k is the von Karman constant (0.41); and $u(z_x)$ is the wind speed at height z_x. Meanwhile, crop roughness length z_0 and zero displacement height d are calculated by:

$$d = 1.1h \ln(1 + X^{0.25}) \quad [31]$$

$$z_0 = \begin{cases} z_{S0} + 0.3hX^{0.5}; & 0 \le X \le 0.2 \\ 0.3h\left(1 - \frac{d}{h}\right); & 0.2 < X \le 1.5 \end{cases} \quad [32]$$

where $X = c_d L$; $\underline{c_d}$ is the mean drag coefficient for individual leaves (0.2); and L is the leaf area index (Choudhury & Monteith, 1988). The aerodynamic resistance between the mean canopy flow and reference height r_a^a is calculated by:

$$\hat{r}_a^a = \frac{1}{k^2 u(z_x)}\left(\ln\frac{z_x - d}{z_0}\right)^2 \quad [33]$$

$$r_a^a = \begin{cases} \hat{r}_a^a/(1 + \varepsilon)^2 & \text{stable, } \varepsilon < 0 \\ \hat{r}_a^a/(1 + \varepsilon)^{3/4} & \text{unstable, } \varepsilon > 0 \end{cases} \quad [34]$$

where

$\varepsilon = 5g(z - d) \cdot (T_0 - T_r)/u(z_x)^2 T_r$; and g is the acceleration due to gravity (9.81 ms^{-2}) (Choudhury & Monteith, 1988).

The mean boundary layer resistance of a crop $r_a^{c,i}$ over the total leaf area index L is calculated by:

$$r_a^{c,i} = \frac{50\alpha}{L} \cdot \left[\frac{w}{u(h)}\right]^{1/2} \cdot \left[1 - \exp\left(-\alpha/2\right)\right]^{-1} \quad [35]$$

where α is the wind speed attenuation coefficient within the canopy; and w is the mean leaf width (Choudhury & Monteith, 1988). The wind speed attenuation coefficient α is determined by:

$$\alpha \approx \frac{0.2Lh}{l_m} \quad [36]$$

where l_m is the canopy mixing length which is determined by assuming square leaves, or:

$$l_m = \left(\frac{3w^2}{2\pi\rho_f}\right)^{1/3} \quad [37]$$

where ρ_f is the leaf area density (Goudriaan, 1977).

A crop's stomatal resistance r_{st}^i is assumed to be related only to PAR (photosynthetically active radiation). This relationship can be described by:

$$\frac{1}{r_{st}^i} = \frac{a_1 \cdot I_{PAR}}{a_2 + I_{PAR}} \quad [38]$$

where I_{PAR} is the PAR irradiance, whereas a_1 and a_2 are the empirically-determined coefficients (Jarvis, 1976). Bulk stomatal resistance is simply determined by:

$$r_s^{c,i} = \overline{r_{st}^i}/L \quad [39]$$

(Thom, 1972; Shuttleworth, 1978; Stannard, 1993).

Soil resistance is assumed to be related only to soil moisture content according to the relationship described by:

$$r_s^s(\Theta) = r_s^s(0)\exp\left(-\varepsilon \cdot \Theta/\Theta_{sat}\right) \quad [40]$$

where $r_s^s(0)$ is the soil resistance when soil is totally dry, as determined by:

$$r_s^s(0) = \frac{\tau\, l}{\varphi_p D_v} \quad [41]$$

where D_v is the molecular diffusion coefficient (2.5×10^{-5} m^2 s^{-1}); τ is the soil tortuosity (2); l is the effective drying soil depth (0.15 m) (Choudhury & Monteith, 1988), and ϕ_p is the total soil porosity, which is calculated as:

$$\varphi_p = 1 - \frac{\text{bulk density}}{\text{particle density } (2.65 \text{ Mg m}^{-3})} \quad [42]$$

and parameter ε is $1/\lambda$, where λ is the pore-size distribution index defined by Brooks and Corey (1964) as:

$$\left(\frac{\psi_e}{\psi}\right)^{\lambda} = \frac{\Theta - \Theta_r}{\Theta_{sat} - \Theta_r} \quad [43]$$

where ψ is the soil matric suction at volumetric water content Θ; ψ_e is the air entry suction; Θ_r is the residual volumetric water content; and Θ_{sat} is the volumetric water content at soil saturation.

Soil

The soil classes represent basic soil properties at a given soil depth, such as bulk density, particle-size distribution (texture), and soil water properties. Users will supply the data on the soil moisture characteristic curve, and based on this curve, properties such as porosity, air entry suction, pore-size distribution index (Brooks & Corey, 1964), and the water amount at a given suction, such as at saturation, field capacity, permanent wilting point, and air dry can be determined. Note that the x-library does not implement a soil water flow model, and consequently, users must always supply the current soil water amount. However, because the x-library is object-oriented and designed to be reusable and extendible, users can actually implement their own soil water flow model and incorporate it seamlessly into the x-library so that the current soil water amount does not have to be manually specified for each simulation run.

FILE INPUT AND OUTPUT

The x-library implements object persistence with the help from the XFile class, where the current state of an object can be stored to or retrieved from a text file. Classes supporting object persistence are those inherited from the base class XObject. Similarly, users can also create or edit the text file which can be used to enter or change an object's attributes (i.e., model parameters) because object persistence is implemented in the text form.

Data on weather are supplied in two forms; daily and hourly. The daily weather properties must include both minimum and maximum air temperatures, vapour pressure, irradiance and wind speed, and these data are stored in a pre-defined format in a text file. Meanwhile, the hourly weather properties are stored in a separate text file and listed in a pre-defined format as well, and these include the air temperature, irradiance, wind speed and vapour pressure.

Support Classes

The support classes (Table 1) are to provide minor, specialised services that are required by other classes. The XDate class, for example, deals with time, and date such as the calculation of the day of year, is used by the weather classes. The exception classes (e.g. Exception, GeneralError and SpecificError) are to support error-handling tasks, and the distributional classes XDist and XCurveFit are to store a two-dimensional array of (x, y) pairs of values. The last two classes can be used to interpolate between the stored pairs of values, where the curve-fitting in XDist is by the cubic spline method, whereas the XCurveFit uses linear interpolation. The XDir class is for directional properties, such as to store the leaf inclination and azimuth angles.

Finally, the factory classes (Gamma *et al.*, 1995) are used to ensure the correct object types are created, depending on the chosen type of radiation model. For the 1-D radiation model, the XRadLayer object must be created along with its support objects, as listed in Table 2a. Likewise for the 2-D radiation model, the XRadCell object must be created with its support objects as listed in Table 2b. Therefore, to ensure the correct object types are always created together, the XFactoryLayer and XFactoryCell objects are used for the 1-D and 2-D radiation model, respectively. These factory classes can thus be regarded as a template or mould to create proper object types. A factory object is used when an object is loaded from a file. For example, the C++ sample source code:

```
XFile fin("c:/data/input.dat");   // 1. file object
XFactoryLayer factory;            // 2. factory object
LOAD ar(&fin, &factory);          // 3. LOAD object
XMicroclimate mc;                 // microclimate object
mc.Load(ar);                      // load object now
```

Specifies loading of an object from file and requires three objects to be created first: the file object representing the data or input file, the factory object, and the LOAD object to hold both the file and factory objects. An object's Load method has two functions: 1) to create the proper objects using the factory object given, and 2) to assign values to the object's attributes from the given file object. Consequently, in the above example, after the call to its Load method, the microclimate object will have its pointers pointing to the correct type of objects, and its objects being pointed to will in turn point to the correct type of objects as well, and so on. The microclimate object will also have the proper values assigned to its attributes from the input file. After loading, the microclimate object in this example is now ready for use, and will implement the 1-D radiation model.

GUI FRONT-END FOR THE X-LIBRARY

As stated, the x-model is a graphical user interface (GUI) front-end for the x-library and it shields users from the implementation and programming details of x-library (*Fig. 4*). As a result, users of the x-model do not necessarily be programmers or they are also not required to understand the x-library framework. The x-model simplifies user input through a consistent and attractive graphical interface. The x-model runs only in the operating system Windows 95 and above, and was developed using MFC (Microsoft Foundation Classes) and compiled with Microsoft Visual C++ 2003. The x-model files occupy approximately 3 Mb disk space.

When users start the x-model, they have to choose to either use the 1-D or 2-D radiation model, after which the user will then input the

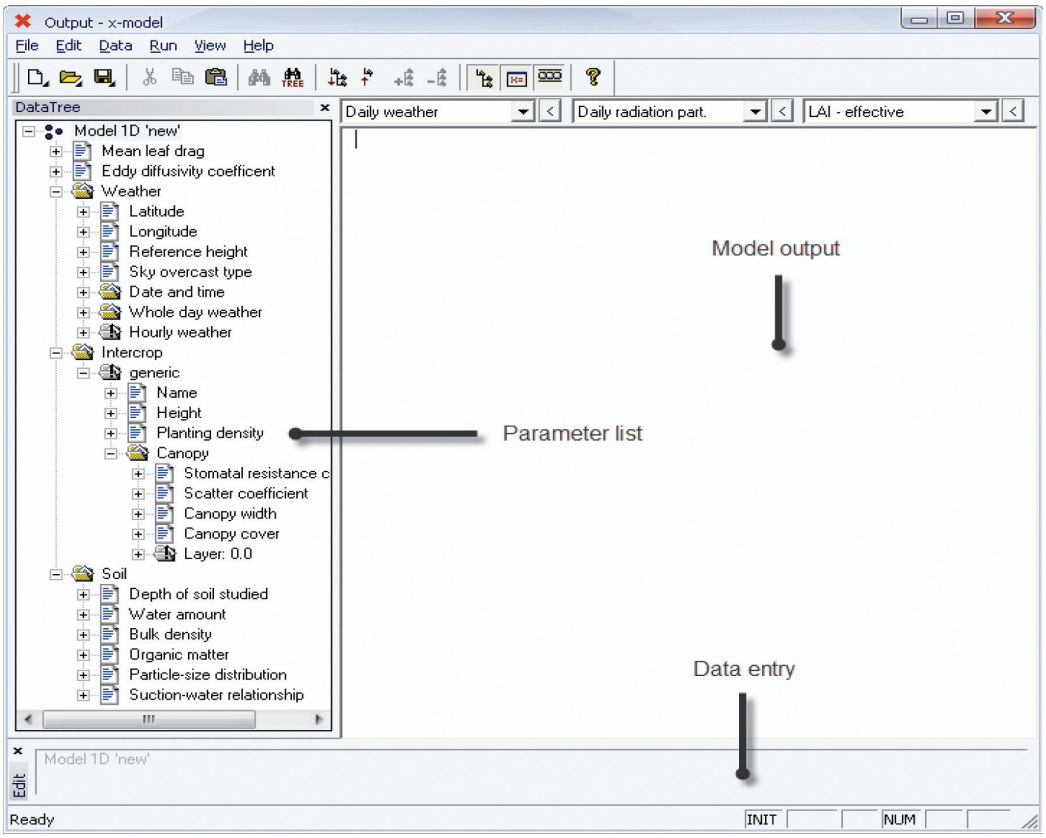

Fig. 4: x-model, the front-end for x-library

required model parameters. The model input can be further divided into four groups, which are referred to as follows: 1) weather, 2) soil, 3) intercrop, and 4) miscellaneous.

Weather Parameters

Weather inputs include site latitude, longitude, hour and day of year to determine the position of the sun, daylength, time of noon, and time of sunrise and sunset. For this purpose, two kinds of weather files are required: one containing daily weather data, and another for hourly weather data. These weather properties are needed to calculate the radiation captured and the various heat fluxes within the system. In addition, the reference height, where these weather properties are measured, is also required. Lastly, the type of sky is also required to determine the amount of diffuse radiation, where on cloudless days, the sky type is of UOC (isotropic), and on cloudy days, the sky type is assumed to be of SOC (Standard Overcast Sky). It is important to note that the UOC and SOC types are as defined by Anderson (1964).

Soil Parameters

Some examples of the soil parameters are bulk density, volumetric water content, and the soil moisture characteristic relationship (suction vs. volumetric water content). Bulk density is needed to determine the total porosity which is used to calculate soil surface resistance. The determination of the soil surface resistance also requires volumetric water content and the soil moisture characteristic relationship.

Intercrop Parameters

The intercrop parameters for the 1-D radiation model are different than the ones for the 2-D radiation model. In particular, the 1-D model requires less complex data on canopy architecture, whereas the 2-D model needs highly-refined data on the individual leaves in the canopy.

Using the 1-D Radiation Model

For the 1-D radiation model, crop height, mean leaf width, and planting density are required for each crop in the intercropping system. In particular, crop height and mean leaf width are needed mainly for aerodynamic resistance calculations. The model also requires input on the canopy properties, such as leaf area index, depth of canopy, and the stomatal resistance coefficients a_1 and a_2, as used in Equation (38). The leaf scattering coefficient is also required to account for the scattering of radiation by the canopy. Meanwhile, the leaf inclination and azimuth densities are required to calculate the G-function. Similarly, to account for the situation where leaves are not randomly distributed but clumped along rows, fractional canopy cover is required to calculate the clump factor.

Using the 2-D Radiation Model

For the 2-D radiation model, input on the planting row direction is needed so that the 2-D network of cells can be arranged perpendicular to the planting row direction. In addition, the 2-D model requires information on the width and height of a cell. For each crop in the intercropping system, crop height, planting density, and planting distance are also required. The planting distance refers to the distance the crop is planted from a reference point (*Fig. 5*). The planting distance enables the model to work out the order of the crop positions in the cell network. As shown in *Fig. 5*, the model assumes that all the individual plants of a given crop species will have identical properties. Just like the 1-D model, the 2-D model also requires information on the stomatal resistance coefficients a_1 and a_2, and the leaf scattering coefficient. Moreover, the model also requires the input on the leaf arch type, which must either be linear or second-degree polynomial

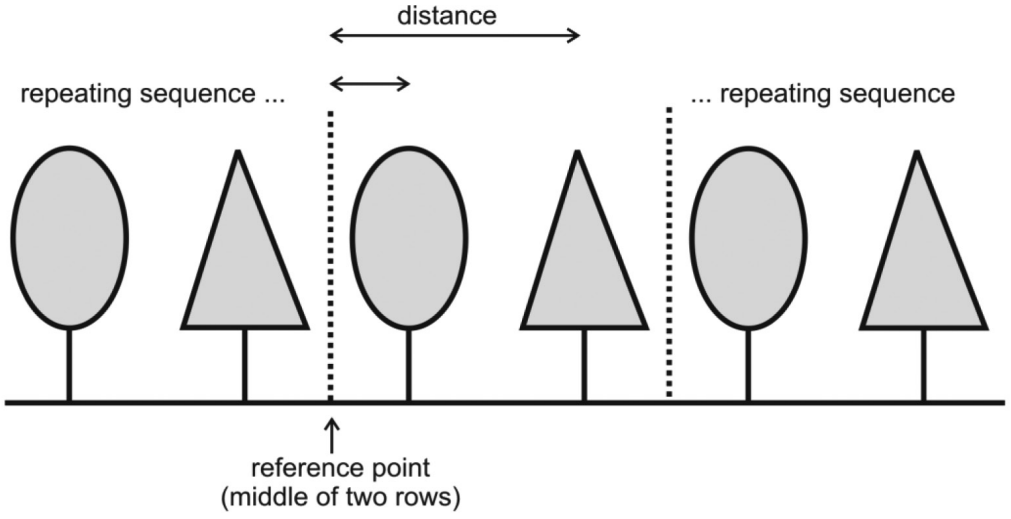

Fig. 5: Planting distance is the distance of a crop to the reference point which is the middle of two planting rows

only. Sunflower leaves, for example, can be set to a linear leaf arch, whereas maize leaves are represented as a polynomial leaf arch. Finally, the model requires information on every leaf. This means that if a plant has 20 leaves, the properties of each leaf must then be supplied as input. For each leaf, the parameters such as the leaf azimuth and inclination angle, and leaf position within the canopy are needed so that the model can determine which leaf section, if any, is encompassed by a given cell. Subsequently, the leaf area density and the G-function can be calculated for the cell based on the encompassed leaf section.

Miscellaneous Parameters

The inputs of the mean leaf drag coefficient and the eddy diffusivity coefficient are needed for heat flux calculations. In addition, the 2-D radiation model requires two other parameters, namely; the number of inclination and azimuth sky intervals, and the number of beams to be "pushed" into each cell in the network. The number of inclination and azimuth intervals correspond to how the whole sky, Ω, is divided to determine the amount of diffuse radiation coming from a particular sky region, as used in Equation (19).

Field Experiment

The simulations of the partitioning of the captured solar radiation and evapotranspiration were done for two types of crops, grown together as an intercrop, namely, maize (*Zea mays*_L. cv. Hudson) and sunflower (*Helianthus annuus* L. cv. Sanluca). The simulations were then compared with the field data obtained by Teh *et al.* (2000) to test the accuracy of the solar radiation and evapotranspiration models.

A full description of the field experiment is given in Teh (2001), as only a brief description is presented in this paper. The maize and sunflower crops were sown together as an intercrop on Sonning Farm, UK (51°27' N and 0°58' W). The field size is 0.13 ha, and the planting densities of maize and sunflower were 30,000 and 15,000 plants ha^{-1}, respectively. Solar irradiance was measured using a sunfleck ceptometer (Decagon Devices Inc., Pullman, Washington, USA; Model SF-80) and plant sap flow using sap flow gauges based on the concept of stem heat balance (Kucera *et al.*, 1977). The field measurements started and ended approximately 30 and 90 days after sowing, respectively.

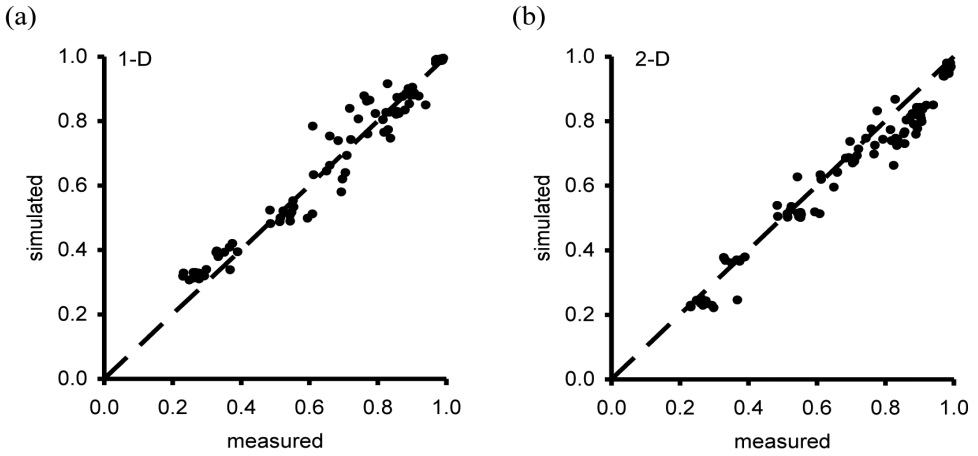

Fig. 6: Comparisons between simulated and measured fraction of total incident radiation intercepted by the maize-sunflower intercrop. Model simulations using the: (a) 1-D model and (b) 2-D model

RESULTS AND DISCUSSION

There was an overall good agreement between the simulated and the measured fraction of the total incident radiation intercepted (*Fig. 6*) for both the 1-D and 2-D radiation models. There was a close clustering of points along the 1:1 line of agreement for both the models. Meanwhile, the mean error for the 1-D radiation model was 0.01 with 95% of errors within -0.09 and 0.11. The mean error for the 2-D radiation model was -0.04 with 95% of errors within -0.13 and 0.06.

In addition, there was also an overall good agreement between the simulated and measured plant transpiration of maize and sunflower (*Fig. 7*). The mean prediction error of transpiration for both the crops was near zero (-0.01 mm h^{-1}) with 95% errors within -0.07 and 0.06 mm h^{-1}. The accuracy of the extended SW equation was not affected by the plant growth stages, but simulated transpiration during the high measured transpiration rates when the measured transpiration for maize and sunflower respectively exceeded 0.15 and 0.40 mm h^{-1}, and tended to be underestimated. This is probably because the x-library did not model the soil water processes rigorously.

Overall, the solar radiation and evapotranspiration models in the x-library showed a good simulation accuracy in modelling the following: 1) the total captured solar radiation, 2) the partitioning of total captured solar radiation between two crops of comparable heights (maize and sunflower), and 3) the partitioning of the transpiration between the two crops. As the x-library is object-oriented, modellers may find it easier to incorporate a plant growth or soil water model into the x-library.

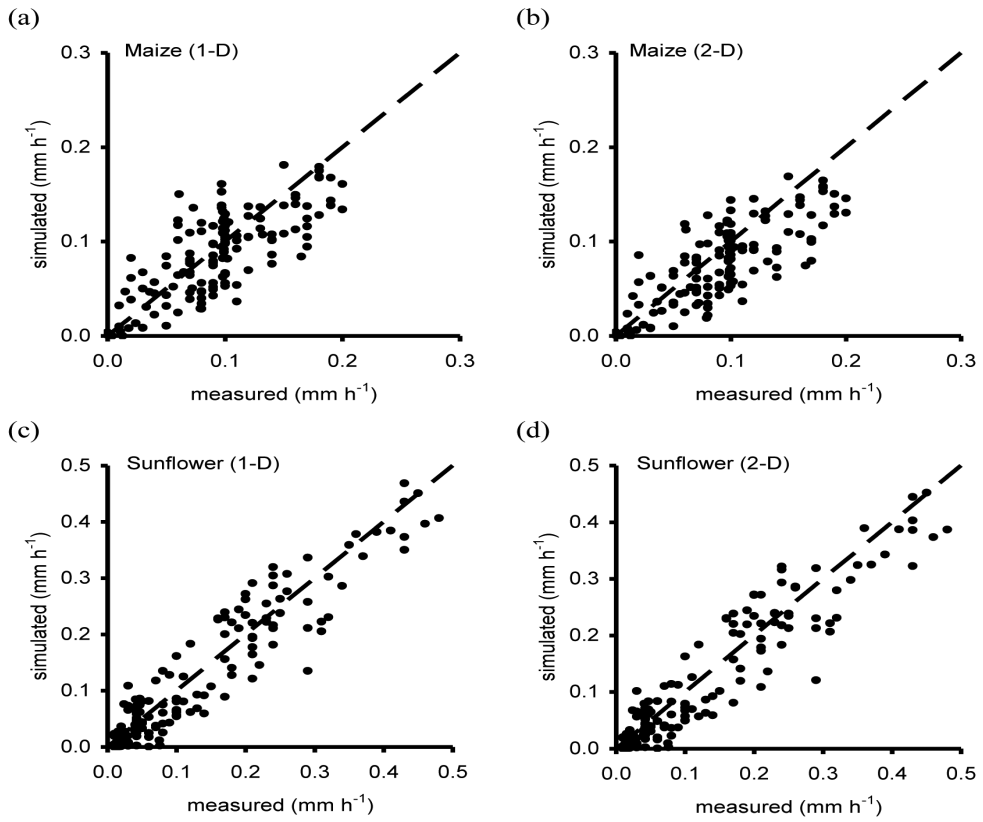

Fig.7: Comparison between simulated and measured transpiration for the maize-sunflower intercrop

CONCLUDING REMARKS

The x-library framework was designed to aid in the development of a solar radiation and evapotranspiration model because it was built using object-oriented principles. The framework simplifies the modification of the existing code and the addition of new code. The model has been found to simulate the partitioning of the captured solar radiation and evapotranspiration with an overall good accuracy. Finally, the x-model was developed as a front-end for the x-library, shielding and assisting users who are not programmers. Both the x-library and x-model are available upon request from the corresponding author.

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Effects of Foliar Applied Copper and Boron on Fungal Diseases and Rice Yield on Cultivar MR219

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ABSTRACT

Long-term intensive cropping on the same piece of land with high-yielding varieties often exhausts the availability of soil micronutrients. Poor management of plant micronutrients has become a hurdle in the effort to increase rice production in Malaysia. The new rice variety, MR 219, was introduced to bring the yield potential up to 10 t ha^{-1}. However, to sustain the high yield, more N-fertilizer input is needed and this will increase incidences of disease. Moreover, nutrients imbalance due to limited micronutrients application may worsen the situation. In this study, two seasons of field experiments were conducted at Sawah Sempadan, Kampung Seri Tiram Jaya, Tanjong Karang (3° 28' 0" North, 101° 13' 0" East) in the off season of 2007 (July 2007 – November 2007) and the main season of 2008 (January 2008 – May 2008), using high yielding cultivar of MR219 to evaluate the effects of copper (Cu) and boron (B) foliar applications on the reduction of fungal diseases, and also to evaluate the effects of foliar Cu and B applications on rice production. Nine combinations of Cu and B treatment at varying levels of Cu (0 – 20 ppm) and B (0 – 20 ppm) were replicated 4 times and applied through foliar spray at three different times, namely, 30, 45 and 60 days after seeding (DAS). The foliar application of Cu and B was found to be able to reduce fungal disease infestation in MR219 rice cultivar and subsequently increase rice yield. Meanwhile, the foliar treatment of Cu and B applied in combination at level T7 (7.53 t ha-^{1}), T9 (7.33 t ha-^{1}), T8 (7.28 t ha-^{1}) and T6 (7.06 t ha-^{1}) produced significantly higher ($P \geq 0.05$) rice yield as compared with the control, T1 (5.75 t ha-^{1}). A significant reduction in disease scoring was also recorded in the experiment where foliar treatment at level T9 (20 ppm Cu + 20 ppm B) cut down 5% of the disease incidence in MR219 rice plant. Rice yield components such as productive tiller m^{-2}, number of spikelets panicle^{-1}, percentage of filled grains and 1,000-grain weight have also shown remarkable increments as a result of the Cu and B foliar treatment.

Keywords: Rice, Micronutrients, Foliar Treatment, Copper, Boron, MR219

INTRODUCTION

Rice (*Oryza sativa* L.) is the main staple food for Malaysians. However, the self-sufficiency level of rice for Malaysia was only 72% in 2006 and the Malaysian government decided to increase its rice self-sufficiency level up to 86% by year 2010 (MARDI, 2006). Most of the granary areas in Malaysia are well-established with irrigation

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systems and farmers practicing double cropping with high-yielding varieties, such as MR219 or MR220 (Ho *et al.*, 2008). However, long-term intensive cropping on the same piece of land with high-yielding varieties has altered the availability of soil micronutrients (Wei *et al.*, 2006). Meanwhile, severe soil micronutrients deficiency has been reported to greatly affect rice production in the main granary areas in Malaysia (Zulkefli *et al.*, 2004).

Sumner (1997) reported that severity of fungal diseases increased in the recent years, with the introduction of high yielding cultivars that require higher fertilizer levels. Fungal diseases, such as brown spot (*Drechslera oryzae*), rice blast (*Pyricularia oryzae*) and sheath blight (*Rhizoctonia solani*), invade the new high yielding cultivar (MR219) and this maybe due to the large amount of N fertilizer application to achieve the targeted yield and also due to the imbalance of N fertilizer distribution between the vegetative and reproductive growth stage (Graham & Webb, 1991). According to Cassman *et al.* (1997), the need for increased nitrogen (N) fertilizer application to maintain high yield has often resulted in greater fungal disease infestation.

Balanced nutrition does not only help to achieve better yield in crop production but also allows plants to protect themselves from new infection (Agrios, 2005; Narayanasamy, 2002). In particular, copper (Cu) and boron (B) are two micronutrients which play great roles in securing rice production but these have been neglected by farmers. Dobermann and Fairhurst (2000) reported that Cu deficiency causing restricted emergence of new leaves in rice reduced tillering and promoted pollen sterility, while deficiency of B in rice also resulted in stunted growth and reduction in the number of panicles.

Copper compound had been developed into fungicides (Bordeaux mixture) in the early time. It has the ability to denature the spores and conidia of fungus and inhibit spores germination (Agrios, 2005). In addition, copper also helps in ligninification which produces primary defence for the plants to resist fungal diseases (Marschner, 1995; Evans *et al.*, 2007). Boron (B) is reported to be involved in keeping cell wall structure and maintaining membrane function (Marschner, 1995). It is believed to improve the strength of the membrane and cell wall with the cross-linked polymer and strengthen the plants vascular bundles which hold back the invasion of pathogens (Stangoulis & Graham, 2007).

Intensive cropping of high-yielding rice cultivars and mismanagement of fertilizer application have resulted in nutrients imbalance which further cause severe fungal infestation and greatly reduce rice production. Providing balanced nutrition in appropriate amount helps to increase rice production and develop resistance towards fungal diseases. Foliar applications of B and Cu serve as a good means, particularly in supplying rice plants with the nutrients that are crucially needed in time. It is believed that foliar applications of Cu and B will help in reducing the incidence of diseases and promoting rice production. Thus, two seasons of field experiments were conducted using high yielding rice cultivar of MR219 to evaluate the effects of the foliar applications of Cu and B in reducing fungal diseases and also to evaluate the effects of foliar applications of Cu and B on rice production.

MATERIALS AND METHODS

Two seasons of the field experiments were conducted at Sawah Sempadan, Kampung Seri Tiram Jaya, Tanjong Karang (3° 28' 0" North, 101° 13' 0" East) during the off season of 2007 (July 2007 – November 2007) and in the main season of 2008 (January 2008 – May 2008) using high yielding cultivar (MR219). The soil series in the field plots is Sedu which is classified as fine, mixed, isohyperthermic family of Typic Sulfaquent. This is an acid sulphate type of soil. This area has been cultivated with paddy for more than 25 years, where farmers widely use chemical fertilizers and pesticides. No additional micronutrients fertilizer is applied other than the subsidized fertilizers given by the Malaysian government. In addition, this area has been experiencing low rice yields with the average production of 4.5 tonnes per hectare,

with severe infections of fungal diseases, such as brown spot (*Drechslera oryzae*) and sheath blight (*Rhizoctonia solani*). Soil sampling and analysis were carried out prior to the field experiment. The soil analysis showed that the experimental area were lower in Cu and B (Table 1), as compared with the critical nutrients range required by rice as reported by Dobermann and Fairhurst (2000) (Table 2).

Thirty six experimental plots consisting of 9 m^2 in area (3 meter wide by 3 meter long) with an additional of 1 meter width planted next to the adjacent plot as buffer zone against spray drift were lined out in the field and arranged in randomized complete block design (RCBD). Copper and B stock solutions were prepared from the laboratory grade of copper sulphate pentahydrate ($CuSO_4.5H_2O$) with 99% purity and sodium pentaborate ($Na_2B_{10}O_{16}.10H_2O$) with 99% purity. Nine combination of Cu and B treatment, namely control or T1 (0 ppm Cu + 0 ppm B), T2 (0 ppm Cu + 10 ppm B), T3 (0 ppm Cu + 20 ppm B), T4 (10 ppm Cu + 0 ppm B), T5 (10 ppm Cu + 10 ppm B), T6 (10 ppm Cu + 20 ppm B), T7 (20 ppm Cu + 0 ppm B), T8 (20 ppm Cu + 10 ppm B) and T9 (20 ppm Cu + 20 ppm B) were replicated 4 times and applied through foliar sprays at three different times (30, 45 and 60 DAS). Spray applications were made using backpack sprayer with nozzles oriented vertical spraying of 500 mL $plot^{-1}$. Nitrogen (N), phosphorous (P) and potassium (K) fertilizers were applied based on the standard recommended rate in all the treatments. Nitrogen fertilizer (150 kg ha^{-1}) as urea was divided into 3 applications; 25% was applied on 15 DAS, 30% on 35 DAS and 55% on 55 DAS, while phosphorous (90 kg P_2O_5 ha^{-1}) was applied as rock phosphate and potassium (150 kg K_2O ha^{-1}) as murate of potash applied as basal fertilizer at 15 DAS. Throughout the plants growth period, adequate plant protection measures were taken to avoid yield loss due to weeds and pests. However, there was no fungicide application throughout the experimental period and this was done to provide natural fungal disease infestation.

The physiological and yield component parameters measured included productive tillers m^{-1}, number of spikelets $panicle^{-1}$, percentage of

TABLE 1
Chemical properties of the soil samples in the experimental site

Chemical Properties	Values	Procedures
Soil pH	4.61	1:1 (soil weight / water volum), pH meter
EC (μScm^{-1})	141.57	1:1 (soil weight / water volum), EC meter
Total C (%)	12.01	Dry combustion
Total N (%)	0.30	Kjedahl method
Exch. P (mg kg^{-1})	37.23	Bray and Kurtz No. 2
Exch. K (mg kg^{-1})	72.36	Shaking (1*M*. NH4OAc, pH 7.0), AAS
Exch. Ca (mg kg^{-1})	293.36	Shaking (1*M*. NH4OAc, pH 7.0), AAS
Exch. Mg (mg kg^{-1})	119.64	Shaking (1*M*. NH4OAc, pH 7.0), AAS
Exch. Zn (mg kg^{-1})	1.11	Mehlich No. 1, ICP
Exch. Mn (mg kg^{-1})	5.27	Mehlich No. 1, ICP
Exch. Cu (mg kg^{-1})	0.12	Mehlich No. 1, ICP
Exch. Fe (mg kg^{-1})	91.50	Mehlich No. 1, ICP
Exch. B (mg kg^{-1})	0.15	Hot water, ICP

TABLE 2
Critical nutrients concentration for rice in soil

Nutrients	Exch. P (mg/kg)	Exch. K (mg/kg)	Exch. Ca (mg/kg)	Exch. Mg (mg/kg)	Exch. Zn (mg/kg)	Exch. Mn (mg/kg)	Exch. Cu (mg/kg)	Exch. Fe (mg/kg)	Exch.B (mg/kg)
Critical nutrients concentration in soil	12-20	15	20	36	1	3 – 30	0.2 – 0.3	4 - 5	0.5

Source: Dobermann & Fairhurst (2000)

TABLE 3
Rice yield (t ha^{-1}) produced as affected by Cu and B treatments at different levels in the two seasons of the field experiment

Treatments (ppm)	Rice Yield (t ha^{-1})	
	1st Season	2nd Season
T1 (0 Cu, 0 B)	5.75 c	5.06 a
T2 (0 Cu, 10 B)	6.83 ab	5.03 a
T3 (0 Cu, 20 B)	6.25 bc	5.15 a
T4 (10 Cu, 0 B)	6.83 ab	5.06 a
T5 (10 Cu, 10 B)	6.90 ab	5.13 a
T6 (10 Cu, 20 B)	7.06 a	5.18 a
T7 (20 Cu, 0 B)	7.53 a	4.95 a
T8 (20 Cu, 10 B)	7.28 a	5.15 a
T9 (20 Cu, 20 B)	7.33 a	5.24 a

In each row, values followed by the same letter (s) are not significantly different ($P \geq 0.05$) by LSD.

filled grain, 1000-grain weight and grain yield. Meanwhile, the productive tillers were measured in the experimental plot using quadrate with an area of 25 cm^2 at 60 DAS. Ten tillers were randomly sampled within the experimental plot at maturity to determine the yield components, such as the number of spikelets per panicle, the percentage of filled grain and 1,000-grain weight. The rice grain yields were obtained by harvesting all the plants in the experimental plot.

At 80 DAS, five spots in each of the experimental plot were randomly selected by using a quadrate. Twenty five leaves were selected randomly in the quadrate and inspected thoroughly; the leaves infected by fungus were marked. The leave areas infected by disease were monitored and the percentage of leaves area infected by fungus in the quadrate were also estimated and recorded as DS according to the method modified from International Rice Research Institute, IRRI (2002). Disease scoring (DS) for the genotype, which represents both disease incidences and symptom severities can be used as an indicator for virus and fungi resistance.

DS can be calculated as:

$$\frac{n(3) + n(5) + n(7) + n(9)}{tn}$$

where,

n(3), n(5), n(7) and n(9) = number of plants showing a reaction in a scale (3), (5) (7), (9) respectively

tn = total number of plants scored.

Data collected were subjected to the analyses of variance (ANOVA), performed using the SAS programme (SAS, 1998) to determine the statistical significance of the effect of the treatments. When the F-values were significant, the Fisher's Least Significant Difference, LSD test was performed for mean comparison. Meanwhile, the relationship and trend between grain yield, disease scoring and other parameters were analyzed by correlation and regression analyses.

RESULTS AND DISCUSSION

Rice Yield

Rice yields produced during the two seasons of the field experiment are shown in Table 3 where significant increase yield was recorded during the 1st season (off season 2007). Treatments with T7 (7.53 t ha^{-1}), T9 (7.33 t ha^{-1}), T8 (7.28 t ha^{-1}) and T6 (7.06 t ha^{-1}) produced significantly higher ($P \geq 0.05$) rice yield as compared with the control, T1 (5.75 t ha^{-1}). However, rice yield in the 2nd season of the field experiment showed no significant difference.

The application of Cu and B was aimed at supplying nutrients which are important in promoting plant growth and grain formation in rice (Dobermann & Fairhurst, 2000). The foliar treatments were found to be able to supply Cu and B timely to rice plant at the critical stage, as these elements were relatively immobile (Dobermann & Fairhurst, 2000; Tagliavini & Toselli, 2005). The additions of micronutrients, such as Cu and B (individually or in combination) were able to increase crop productions (Heitholt *et al.*, 2002; Zulkefli *et al.*, 2004; Dordas, 2006; Yang *et al.*, 2009).

The applications of Cu and B to the plant through foliar application were found to have enhanced photosynthesis and resulted in better plant growth and grain formation. Copper is needed in photosynthesis and respiration. It also plays a critical role in the formation of pollen and fertilization in rice (Dobermann & Fairhurst, 2000). Gupta *et al.* (2008) reported that high N supply to high yielding crop may accentuate Cu deficiency due to the lower availability of Cu in plants of high N nutritional status. On the other hand, B plays a key role in carbohydrate metabolism, sugar transport and pollen viability in rice (Dobermann & Fairhurst, 2000). Boron has long been identified as one of the major constraints for grain crop production in the world (Rerkasem & Jamjod, 2004; Rerkasem *et al.*, 2004). In the context of Malaysia, Zulkefli *et al.* (2004) have documented B deficiency as a limitation for achieving high yield in major rice planting areas. In particular, the application of B is able to enhance grain production significantly

(Rerkasem & Jamjod, 2004; Rerkasem *et al.*, 2004; Zulkefli *et al.*, 2004). The foliar application of B has been proven to be the most effective method for countering B deficiency in standing crop (Somani *et al.*, 2008).

Rice yield obtained in the 2nd season of the field experiment showed no difference in all the treatments, while rice yield in the 2nd season of the field experiment was also lower than that of the 1st season. This was due to the unfavourable field condition in that particular season (January 2008 – May 2008) where rice diseases infected the entire experimental area. Moreover, the lack of field maintenance by the neighbouring farmers resulted in severe disease infestation in the experimental plot (Pers. Comm. Encik Muhammad Jani Bin Rasimon). Plants in the experimental plot where no fungicide was applied were greatly affected. Copper and B treatments were not able to perform their role in reducing fungal infestation.

The ANOVA showed that the treatments applied in the 2nd season of the experiment resulted in no significant difference in terms of rice yield. Thus, other parameters that contributed to the yield components for the 2nd season of field experiment were not discussed.

Physiological and Yield Component Parameters

The results for the physiological and yield component parameters obtained from the field experiments showed a similar trend where the foliar treatment with 20 ppm Cu and 20 ppm B recorded a significantly better yield (Table 4).

The treatments with 20 ppm Cu and 20 ppm B produced significantly higher number of productive tillers m^{-2} (672.0)($P \geq 0.05$) during the 1st season of the field experiment as compared with the control plot (589.33) (0 ppm Cu and 0 ppm B). Tillers production is greatly affected by the availability of Cu. Copper deficient plant always shows a decrease in the numbers of tillers formed (Dobermann & Fairhurst, 2000). Foliar applications are able to timely supply adequate amount of Cu (Tagliavini & Toselli, 2005) during the critical period of tillering to panicle growth stage to correct Cu deficiency and enhance tillers formation (Dobermann & Fairhurst, 2000). Rerkasem and Jamjod (2004) reported that low B availability could reduce tiller numbers in wheat. Meanwhile, Boron plays an important role in accelerating the formation of panicles in rice plants. Dobermann and Fairhurst (2000) reported that B deficiency, particularly at the panicle formation stage, would greatly reduce the formation of panicles in rice plant.

Rice plants treated with T9 (20 ppm Cu and 20 ppm B) produced significantly higher numbers of spikelets $panicle^{-1}$ (130.43) ($P \geq 0.05$). This demonstrates the importance of Cu and B in enhancing grain formation in rice. Dobermann and Fairhurst (2000) also reported that both Cu and B affected pollen viability in rice plant. On the contrary, copper deficiency reduces seed production in wheat, and this is mainly due to male sterility rather than decrease in photosynthetic CO_2 fixation (Gupta *et al.*, 2008). Moreover, B is also associated with male sterility and grain set failure in grain crops, particularly in wheat (Rerkasem & Jamjod, 2004; Rerkasem *et al.*, 2004). Rerkasem and Jamjod (2004) found that B deficiency impaired the function of anthers and resulted in severe reduction in yield. Other than that, B deficiency also depresses pollen germination and the fertilization process (Cheng & Rerkasem, 1993).

Plants treated with T9 (74.73%) and T8 (73.70%) recorded higher percentages of filled grain as compared with the other treatments. This greatly affects the total grain yield because higher grain filling rates are required to secure maximum yield production. A combination of Cu and B successfully increased grain filling in rice. Both Cu and B play important roles in photosynthesis and respiration, carbohydrate metabolism and sugar transport in rice plant (Dobermann & Fairhurst, 2000). Khush and Peng (1996) stated that translocation and accumulation of photosynthate (carbohydrate) produced in leaves and stems into the grains are prerequisites for higher grain filling rates. Thus, the applications of Cu and B via foliar were able to increase grain filling by more than 5% in MR219.

TABLE 4
Physiological and yield component parameters as affected by different levels of Cu and B treatments in the two seasons of the field experiment

Treatment (ppm)	Productive Tillers m^{-2}		Number of Spikelets $Panicle^{-1}$		Filled Grain (%)		1,000-Grain Weight (g)	
	1st Season	2nd Season	1st Season	2nd Season	1st Season	2nd Season	1st Season	2nd Season
T1 (0 Cu, 0 B)	589.33 b	405.33 a	113.50 b	130.40 a	69.46 e	66.33 a	25.09 cd	24.56 ab
T2 (0 Cu, 10 B)	618.67 ab	418.67 a	118.33 b	116.60 a	70.09 ed	64.66 abc	25.19 bcd	24.98 ab
T3 (0 Cu, 20 B)	629.33 ab	422.67 a	119.55 b	115.18 a	70.68 d	61.39 abcd	24.94 d	24.54 ab
T4 (10 Cu, 0 B)	584.00 b	389.34 a	115.70 b	122.70 a	69.68 e	56.99 d	24.71 d	23.50 b
T5 (10 Cu, 10 B)	630.67 ab	428.00 a	117.18 b	120.58 a	72.18 c	56.77 d	25.10 cd	25.13 a
T6 (10 Cu, 20 B)	633.33 ab	412.00 a	117.95 b	130.23 a	73.57 b	60.75 bcd	25.53 abc	24.91 ab
T7 (20 Cu, 0 B)	636.00 ab	418.67 a	115.23 b	115.35 a	72.55 c	66.05 ab	25.57 abc	24.95 ab
T8 (20 Cu, 10 B)	646.67 ab	421.33 a	113.13 b	125.28 a	73.70 ab	63.90 abc	25.65 ab	24.93 ab
T9 (20 Cu, 20 B)	672.00 a	400.00 a	130.43 a	123.60 a	74.73 a	60.19 dc	25.82 a	24.96 ab

In each row, values followed by the same letter (s) are not significantly different ($P \geq 0.05$) by LSD.

The response of rice cultivar MR219 to the Cu and B treatments with respect to 1000-grain weight was significantly higher in T9 (25.82 g) as compared to the other treatments (Table 4). High concentrations of Cu and B (T9, T8, T7 and T6) significantly influenced the 1000-grain weight of rice planted in the 1st season of the field experiment.

Disease Scoring

Disease scoring (%) showed a significant difference among the treatments in the 1st season of the field experiment (*Fig. 1*). Treatment T9 (24.30%) significantly reduced the number of diseased plants as compared to the others. Meanwhile, control T1 (29.65%) found to be the highest in the number of affected plants in the 1st season of the experiment.

The combined foliar treatments of Cu and B demonstrated a significantly suppressing effect on fungal infestation up to a certain limit. Rice plants with minute amount of Cu and B treatments could sustain and produce high yield even without fungicides treatments. During disease outbreak (2nd season of field experiment), the Cu and B treatments were not able to sustain the incidence of disease which remained high, and the rice yields were also badly affected.

The reduction of disease severity in rice was resulted from the biocidal effect of Cu and the combined effect of Cu and B as essential nutrients. Copper and B involved in the physiological and biochemical processes allow plants to response to pathogens invasion (Marschner, 1995; Dobermann & Fairhurst, 2000; Agrios, 2005; Evans *et al.*, 2007; Stangoulis & Graham, 2007). As an important component of fungicides, copper is able to denature the spores and conidia of fungus, which subsequently inhibit spores germination (Montag *et al.*, 2006).

Other than that, Cu helps in promoting lignification in plants to develop plants primary defence mechanism against fungal invasion (Marschner, 1995). Evans *et al.* (2007) stated the effect of Cu in promoting the formation of lignin and this resulted in reduced fungal diseases in different plant species. Similarly, Brown *et al.* (1995) also demonstrated the effect of Cu in enhancing the cross linking of cell wall

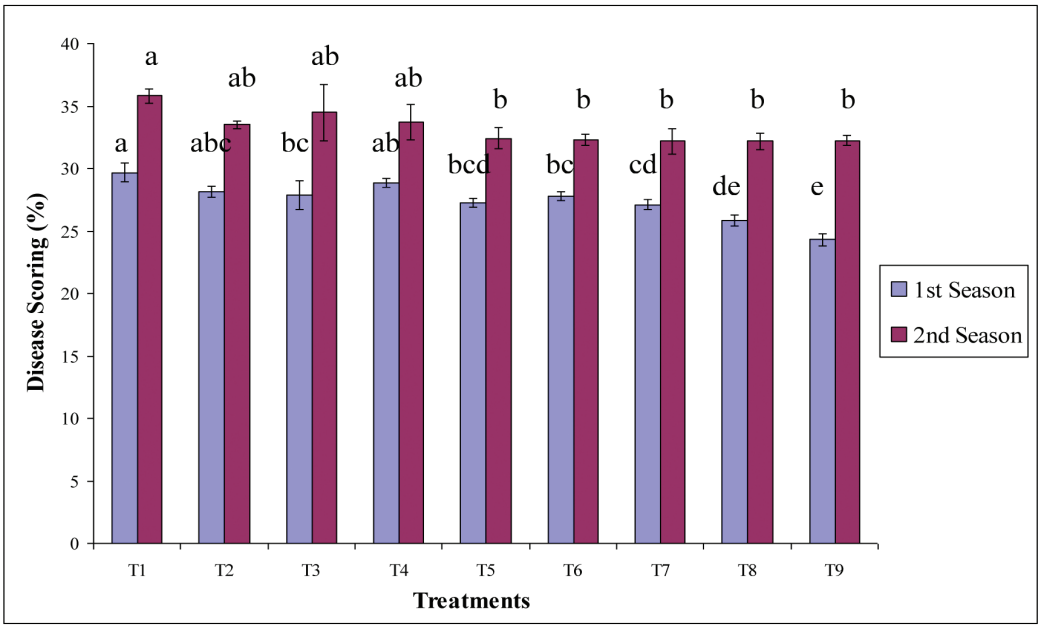

Fig. 1: Disease Scoring (%) as affected by Cu and B treatments at different levels in the two seasons of the field experiment

components and controlling fungal diseases. Modafar and Boustani (2001) revealed that the enhancement of cell wall bound phenolics compound and lignin content in plant tissues promoted host defence against fungus diseases.

Boron is responsible to maintain the cell wall structure, the membrane function and supporting metabolic activities (Bolaños *et al.*, 2004). In addition, Dobermann and Fairhurst (2000) also noted the function of B in enhancing lignin formation in rice. Stangoulis and Graham (2007) reported the importance of B in decreasing fungal diseases in various kinds of corps. The lack of B can cause distortion of cell wall structure and also inhibit biosynthesis of lignin in plants (Marschner, 1995). The foliar treatment of B has also been reported to cut down the number of lesions and reduce the severity of tan spot in wheat through its involvement in physiological and biochemical processes of the plants (Simoglou & Dordas, 2006).

Relationship between Plant Parameters

The coefficient of correlation analysis between rice yield, disease scoring and other yield parameters, such as productive tillers m^{-2}, number of spikelets $panicle^{-1}$, percentage of filled grain and 1000-grain weight, are shown in Table 5.

TABLE 5
Correlation coefficients (r value) between some rice parameters in the field experiment

Plant Parameters	Rice Yield
	Correlation Coefficients
Productive Tillers m^{-2}	0.3028 ns
Number of Spikelets $Panicle^{-1}$	0.0007 ns
Percentage of Filled Grain	0.5392 **
1,000-Grain Weight	0.4646 **
Disease Scoring (%)	- 0.4829 **

ns = Not Significant ** = Highly Significant

A significant correlation exists between rice yield and other parameters such as the percentage of filled grains, 1,000-grain weight and disease scoring (DS). However, disease scoring (DS) is negatively associated with the rice yield. Highly significant correlation exists between the percentage of filled grains and 1,000-grain weight with rice yield, and these indicate the importance of the parameters in promoting rice production. Meanwhile, a positive correlation between these parameters and rice yield is attributed to the improved grain filling of the treatments that contributed to these characteristics. Higher grain filling characteristics indicate better grain weight and density. The negative association between disease scoring (DS) with rice yield showed that rice production is greatly affected by disease incidence.

CONCLUSION

The findings of this study have shown that the foliar applications of Cu and B are able to enhance rice production by 27% under field condition. The yield increment was due to a higher percentage of grain filling and heavier 1,000-grain weight. Moreover, the foliar treatment has also been found to effectively reduce disease incidence by 5% and this is believed to be due to the biocidal effect of Cu and the enhancement of the physiological properties of the plants itself, such as better lignification and fortified cell membrane which is the primary defence mechanism to build up plants resistance. However, the foliar treatment of Cu and B should be applied together with proper plant protection measures, such as fungicide application, to ensure better plant growth and enhance rice production.

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Level of Polychlorinated Biphenyls (PCBs) in Selected Marine Fish (pelagic) from Straits of Malacca

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ABSTRACT

Fish is a good source of protein, supply important vitamins and other essential nutrients including essential fatty acids (EFA), the EPA and DHA which help to reduce risk of death from coronary heart diseases. However, diet and food of animals' origin are the most predominant sources of polychlorinated biphenyls (PCBs) to human which accounts to over 90%, with fish as one of the major routes of contaminants in human body. PCBs are a group of extremely stable aromatic chlorinated compounds which are relatively resistant to biological degradation and very persistent in the environment. This study has identified the type and level of 12 congeners of PCBs that are most toxic to humans. The maximum permitted level of PCBs in muscles meat of fish and fishery products is 4 pg/g, as recommended by World Health Organization (WHO) using the WHO-TEFs. Meanwhile, the highest amount of PCBs concentration was in *Rastrelliger kanagurta* (Indian mackerel), with the level of PCBs at 1.37 pg/g wet weight. Other species like *Scomberomorus guttatus* (Spanish mackerel), *Pampus argenteus* (Silver pompret), *Megalapsis cordyla* (Hardtail scad), *Eleutheronema tradactylum* (Fourfinger threadfin) and *Chirocentrus dorab* (Dorab wolfherring) showed PCBs levels ranging from 0.35 pg/g to 1.05 pg/g wet weight. Thus, the PCBs in all the samples were below the permitted level. It can be concluded that the studied pelagic fish are safe to consume. Although the levels were not high, it is still important to set limits for the PCBs in fish and shellfish species so as to make a better estimation of the risk of exposure to human through dietary intake of fish, specifically fatty fish to meet nutritional requirement for EPA and DHA.

Keywords: Fish, Polychlorinated biphenyls, PCBs, pelagic

INTRODUCTION

Polychlorinated biphenyls (PCBs) are a group of extremely stable aromatic chlorinated compounds which are relatively resistant to biological degradation and very persistent in the environment. Two important events involving direct overexposure of human to PCBs occurred

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in Japan (Yusho) in 1968 and Taiwan (Yu-Cheng) in 1979. In both cases, rice oil contaminated with PCBs was ingested and caused illnesses to individuals consuming the rice oil (Yu *et al*., 1991) such as various somatic complaints, low birth weights, chloracne and hyperpigmentation, especially of newborn children (Kuratsune *et al*., 1972). There are many reported biological and toxicological effects of these compounds and their impacts on human health had become an extremely controversial issue in environmental toxicology, endocrinology and carcinogenesis. Consequently, the marketing and use of PCBs have been restricted in European Union (EU) through Directive 85/467/EC, while the use of PCBs in some European countries has been banned since as early as 1973 (Boscolo *et al*., 2007). Generally, PCBs accumulate in the environment and food chain (Bocio *et al*., 2007). There are 209 PCB congeners altogether, but only 12 of the non-*ortho* and mono-*ortho* PCBs are identified as dioxin-like by World Health Organization (Van den Berg *et al*., 2006). The principal sources of PCBs encountered in the environment include open burning or incomplete combustion, vaporization of PCBs in open applications, accidental spills or leakages of PCBs in close system applications and disposal into sewage system (Connell, 2005). One of the strongest tendencies to accumulate PCBs from water and food source is aquatic organisms. Diet and food of animal origin are the most predominant sources of PCBs to human, and these account to over 90% (Svensson *et al*., 1991). Although fish and shellfish generally represent only a small percentage (10%) of these contaminants, these foods are some of the major routes of the contaminants in the human body (Harisson *et al*., 1998).

Fish can be classified according to the environment where they live in, i.e. whether from freshwater or marine, pelagic or demersal. Pelagic fish are ones that live in the surface of ocean. Naturally, this variation in habitat leads to a wide variance in the character of the fish. In Malaysia, Indian mackerel, Silver pompret, Fourfinger threadfin, Hardtail scad are some examples of the pelagic fish (Abdul Majid, 2004). In addition, the fat contents in fish are also depending on the habitats. Besides, fish is also commonly classified as white fish and oily fish, depending on the parts of the body they store fat in. The white fish store their fat in the liver, while oily fish store their fat in the liver and throughout their bodies (Brown, 2008).

Fish is a good source of protein. Fish protein is of high-quality, providing 17% of the total animal protein and 6% of all the protein consumed by humans. Fish also supply important vitamins and other essential nutrients (Torpy *et al.,* 2006; Domingo *et al.*, 2007). Nutritionist has suggested intake of 35g or more fish daily (Daviglus *et al.,* 1997) and two fatty fish-meals per week (Siscovick *et al.,* 1995) to reduce relative risk of death from coronary heart diseases. Besides protein, fish is also high in essential fatty acids (EFAs) known as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are important to human diet.

The presence of PCBs in some fish and seafood can be sufficiently high, and this poses a potential health risk to consumers, particularly susceptible adults, foetuses, neonates, and developing infants (Burger & Gochfeld, 2008). Various investigations have been conducted over the past few years to quantitatively compare the risks of exposure to chemical pollutants in fish with omega-3 fatty acids associated health benefits of fish consumption (Sidhu, 2003). For example, Hites *et al*. (2004) determined the concentrations of various organic contaminants including PCBs in salmon. The results of the risk analysis indicated that the consumption of farmed Atlantic salmon might pose health risks that detracted from the beneficial effects of consuming fish. Salmon is one of the fatty fish included in the list of species that contain high omega-3 fatty acids, EPA and DHA. According to World Health Organization (WHO), toxic equivalents using the WHO-TEFs, the maximum level of PCBs is 4 pg/g for muscles meat of fish and fishery products and products thereof with the exception of eel (Van den Berg *et al*., 2006).

Up to this date, varied levels of polychlorinated compounds (PCBs together

with dioxins and furans) in various fish and shellfish species have been widely published from different countries, including Tunisia (Masmoudi *et al*., 2006), Spain (Bocio *et al*., 2007), Ireland (Tlustos *et al*., 2006), Scotland, UK (Jacobs *et al*., 2002) and US (Jensen & Bolger, 2001). In Asia, however, reported data on these contaminants are still lacking and sparse. Therefore, this study was carried out to obtain data on the level of PCBs in fish from this region, particularly Malaysia.

As for Malaysia, specifically along the Straits of Malacca, the level of PCBs could be potentially high due to the various industrial activities along the West Region of Peninsular Malaysia, as well as the Straits, being one of the busiest routes in the world. Therefore, this study is important for identifying and quantifying the type and the amount of polychlorinated biphenyls (PCBs) in commonly consumed marine fish caught along the strait. In addition, data of this study are also useful to economically protect local and export markets of fish industry in Malaysia. Moreover, it gives important information on the safety aspect of local fish as consumers nowadays are aware on the beneficial intake of fish, particularly for its high level of EFAs.

MATERIALS AND METHODS

Chemical

The reagents used were pesticide residue grades, such as hexane, dichloromethane, toluene from Fisher Scientific, Leicestershire, UK.

Instrumentation

Accelerated solvent extraction (ASE200, Dionex Corp., Sunnyvale, CA, USA), Fluid Management System (FMS, Inc, Waltham, MA, USA), High-resolution gas chromatography/ high-resolution mass spectrometry (MAT 95 XL, Agilent 6890 Series, USA), Rotary Evaporator (BUCHI Labortechnik, Flawil, Switzerland).

Sample Collection

In this study, a stratified sampling method was used to collect the fish samples. In this sampling method, fish samples were collected from defined strata of the known fish landing areas along the Straits of Malacca. Within each landing site, the samples were taken randomly. Stratified sampling is the most suitable method for a database study (Greenfield & Southgate, 2003). Fresh fish samples were collected from 10 identified fish landing areas, along the Strait of Malacca, which were further divided into 3 regions; namely North region (Kuala Perlis, Kuala Kedah, Teluk Bahang and Batu Maung), South region (Port Dickson, Malacca and Muar) and Middle region (Kuala Selangor, Manjung Utara and Matang) (*Fig. 1*). The collection of the samples at each region was carried out twice in August to November 2008. The fish samples were freshly collected from the landing sites with the help from the Fisheries Development Authority of Malaysia (FDAM/ LKIM). The fresh samples of fish were immediately dipped in a mixture of water and ice to block any digestive and unfavourable changes. From the collection site to the laboratory, the samples were transferred in polystyrene boxes containing ice and transported on the same day at a refrigerated temperature (4^{o}C).

Fish Samples

There were 6 species of fish collected; namely, *Rastrelliger kanagurta* (Indian mackerel), *Scomberomorus guttatus* (Spanish mackerel), *Pampus argenteus* (Silver pompret), *Megalapsis cordyla* (Hardtail scad), *Eleutheronema tradactylum* (Fourfinger threadfin), and *Chirocentrus dorab* (Dorab wolfherring). The selection of the fish samples was based on work by Osman *et al*. (2001), with most of the species were those preferred by the local consumers.

Preparation of the Sample

Upon arrival in the laboratory, the collected samples were individually measured for their

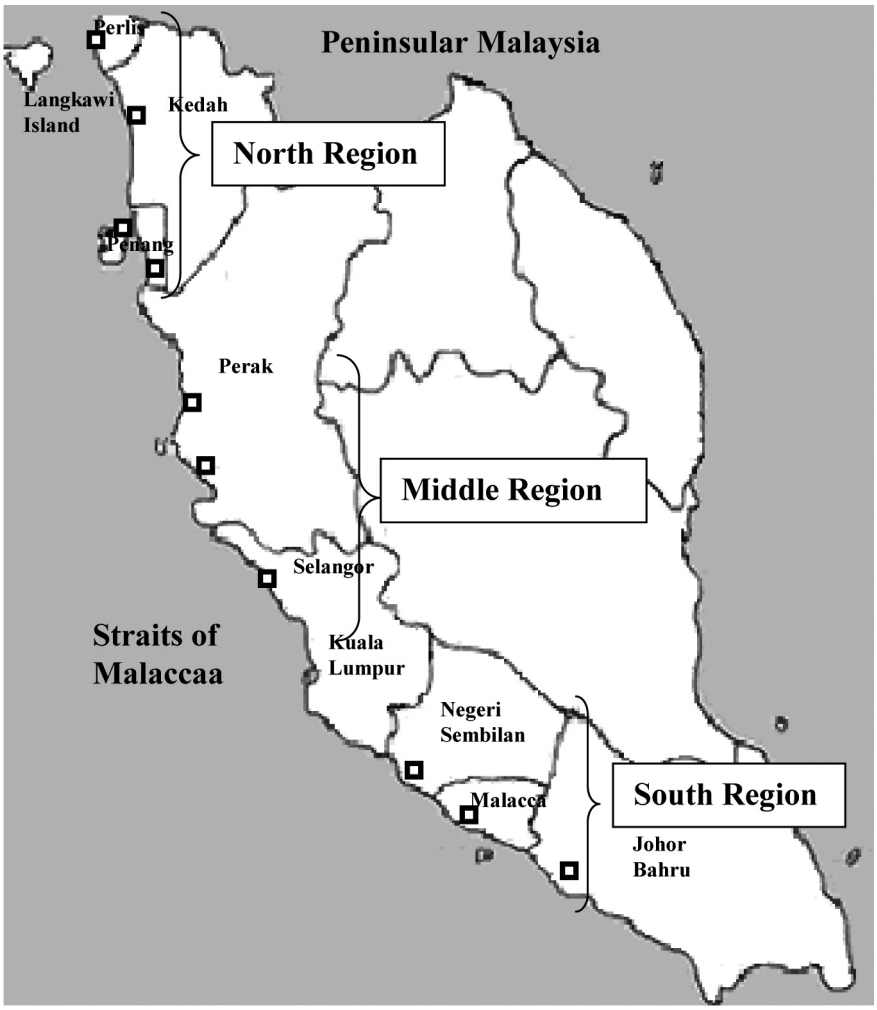


Fig. 1: Location of the sampling sites

length and weight. Fish samples were gutted, viscera removed, beheaded, washed and fillet before frozen. All the samples were kept at -75°C without any prior treatment. Before the analysis, the composite sample of each species in different regions was prepared by homogenously mixing and grinding the prepared samples using a blender (National, Petaling Jaya, Malaysia). All the composite samples were packed into polyethylene (PE) covered cup, stored in a freezer at -20°C and analyzed within a week.

Fat Extraction and Sample Clean-up

Twenty (20µl) of 4937 PCBs internal standard (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was injected into ten grams of homogenized wet muscle tissue and extracted by Accelerated Solvent Extraction (ASE) 200 (Dionex Corp., Sunnyvale, CA, USA). Meanwhile, about 7g of hydromatrix was added and mixed together with the sample. After that, the sample mixture was put in a microwave oven (Khind, Petaling Jaya, Malaysia) for drying for about 2 minutes. The dried sample was then put into ASE extraction cell (size

TABLE 1
List of the samples with narrow range of weight and length

Regions	Local name	Common name	Scientific name	n	Weight (g) (min-max)	Length (cm) (min-max)
North	Bawal putih	Silver pomfret	*Pampus argentus*	5	131 – 243	15 - 25
	Cencaru	Hardtail scad	*Megalapsis cordyla*	8	154 – 254	17- 22
	Kembung	Indian mackerel	*Rastrelliger kanagurta*	10	42 – 104	16- 19
	Senangin	Fourfinger threadfin	*Eleutheronema tetradactylum*	4	210 - 379	30- 31
South	Cencaru	Hardtail scad	*Megalapsis cordyla*	8	209 - 256	25- 27
	Parang	Dorab wolfherring	*Chirocentrus dorab*	2	200 - 900	40 - 71
	Tenggiri papan	Spanish mackerel	*Scromberomorus guttatus*	2	200 – 450	30 - 42
Middle	Cencaru	Hardtail scad	*Megalapsis cordyla*	9	63 – 230	28- 30
	Kembung	Indian mackerel	*Rastrelliger kanagurta*	12	22 – 54	13- 16
	Senangin	Fourfinger threadfin	*Eleutheronema tetradactylum*	6	158 – 245	28- 30

TABLE 2
Fat contents (g/100g) and total PCBs (WHO-iTEQ pg/p wet weight) in the pelagic fish from different regions along the Strait of Malacca

Regions	Species (common name)	Fat (g/100g)		Mean	Variation (%)	WHO-iTEQ (pg/g)		Mean	Variation (%)
		T1	T2			T1	T2		
North	*Megalapsis cordyla* (Hardtail scad)	0.6	1.0	0.80	35.36	0.73	0.35	0.54	49.76
	Eleutheronema tradactylum (Fourfinger threadfin)	2.0	2.7	2.35	21.06	0.37	0.55	0.46	27.67
	Rastrelliger kanagurta (Indian mackerel)	1.0	3.1	2.05	72.44	1.37	0.35	0.86	83.87
	Pampus argenteus (Silver pompret)	3.6	3.7	3.65	1.94	0.35	0.53	0.44	28.93
South	*Megalapsis cordyla* (Hardtail scad)	0.4	3.1	1.75	109.10	0.35	0.50	0.43	24.96
	Chirocentrus dorab (Dorab wolfherring)	3.8	3.5	3.65	5.81	0.54	0.68	0.61	16.23
	Scomberomorus guttatus (Spanish mackerel)	3.2	5.5	4.35	37.39	0.64	0.72	0.68	8.32
Middle	*Megalapsis cordyla* (Hardtail scad)	2.8	3.5	3.15	15.71	1.05	0.77	0.91	21.76
	Eleutheronema tradactylum (Fourfinger threadfin)	1.8	3.3	2.55	41.59	0.47	0.38	0.43	14.97
	Rastrelliger kanagurta (Indian mackerel)	3.3	6.7	5.00	48.08	0.35	0.35	0.35	0.00

% variation was calculated based on the following formula: (SD/Mean) × 100
TI is Trip 1 (12 August 2008 to 9 September 2008)
T2 is Trip 2 (15 October 2008 to 12 November 2008)

33) and the extraction process was carried out for 20 minutes. After the ASE extraction, the solvents used were removed by means of a rotary evaporator **(**BUCHI Labortechnik, Flawil, Switzerland**)** that was at 500 Mbar, at 40-50°C for 20-30 minutes. The fat fraction extracted was determined gravimetrically.

After fat extraction, eluents (fat and hexane) were cleaned up using acid/base modified silica gel, alumina, and graphitized carbon column chromatography for about 1 hour. Later, hexane was removed from the PCBs eluent using a rotary evaporator **(**BUCHI Labortechnik, Flawil, Switzerland**)**. Then, 20µl of 4798 PCBs external standard (Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was added into the sample in a small vial and mixed well. The process was continued by drying the mixture of the samples on a heating block at 70°C with nitrogen gas until the amount of the samples reached 10µl. After that, the sample was analyzed using high-resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). Later, the samples were analyzed for 12 dioxin like co-planar PCBs; PCBs 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189. The sample preparation procedures, analytical techniques, and quality control strategies described are as defined in the US EPA's Method 1613 and by Ferrario *et al*. (1996, 1997).

Calculation of Toxicity Equivalents (iTEQ)

Toxicity equivalent (iTEQ) was calculated using the procedure developed by WHO (2005). The toxicity of PCBs congeners was expressed using toxic equivalence factors (TEFs) representing the relative toxicity of the compound being measured to the most toxic PCBs 126 congener, with TEF value of 0.1. The TEQ of PCBs was calculated by multiplying the analytical determined concentration of each congener by its corresponding TEF.

RESULTS AND DISCUSSION

Table 1 shows details of 6 pelagic species of fish analyzed in this study. Knowledge on each species size is important as previous literature has stated that the occurrence of chemical contaminants and bioaccumulation is related to length, weight and age of fish (De Marco *et al.*, 2006). Furthermore, certain ecological factors, such as season, place of development, nutrient availability, temperature and salinity of the water may also contribute to the inconsistency of contaminants in fish tissue (Tuzen, 2003). Table 2 shows the summary information on the level of fat and PCBs measured in the pelagic species of fish available from the fishing landing areas during the sampling period. The value of the results was expressed as total WHO-iTEQ in pg/g wet weight samples for PCBs and g/100g of the wet weight for the fat content. There were 12 congeners of PCBs detected and reported for each sample. Meanwhile, WHO toxic equivalent factors (WHO-TEFs-2005) were used to calculate the TEQ of each sample. The results consisted of 6 different fish species of 20 samples. The fat content varied from one species to another. In the present study, the highest fat content was observed in *Rastrelliger kanagurta* (Indian mackerel), i.e. in the range of 2.05-5.00 g/100g wet weight sample. This was followed by *Megalapsis cordyla* (Hardtail scad) which contained low fat in the range of 0.80-3.15 g/100g of the wet weight sample. Other species, such as *Eleutheronema tradactylum* (Fourfinger threadfin), *Pampus argenteus* (Silver pompret), *Chirocentrus dorab* (Dorab wolfherring) and *Scomberomorus guttatus* (Spanish mackerel) showed fat contents of 2.35-2.55 g/100g, 3.65 g/100g, 3.65 g/100g and 4.35 g/100g, respectively. Osman *et al.* (2001) reported the fat content of *Rastrelliger kanagurta* (Indian mackerel) was 4.54 g/100g, and this is within the range obtained in this study. As for other species, such as *Pampus argenteus* (Silver pompret), Osman *et al*. (2001) reported that it contained 2.91 g/100g, the amount which is much lower than the value obtained in the present study. On the other hand, Tee *et al.* (1997) reported the fat content of *Pampus argenteus* (Silver pompret) at 4.0 g/100g, and this is much higher than the value stated in this study. The differences in fat content observed could be due to variations in the

sub-species, seasons, geographical regions, age and maturity that might influence the fat of fish (Osman *et al*., 2001). For instance, the variations within the species between Trip 1 and Trip 2 vary greatly between 1.94% to less than 50%, except for *Megalapsis cordyla* (Hardtail scad) and *Rastrelliger kanagurta* (Indian mackerel), which clearly indicated that the fat content variation in one species was affected by various factors.

For the North region, the highest value of the total PCBs in Trip 1 was obtained in the *Rastrelliger kanagurta* (Indian mackerel) species, at the concentration of 1.37pg/g in the wet weight of sample. In contrast, the lowest value was obtained in *Pampus argenteus* (Silver pompret) species at 0.35pg/g wet weight. As for *Megalapsis cordyla* (Hardtail scad) and *Eleutheronema tradactylum* (Fourfinger threadfin), the levels obtained in this study were 0.73 pg/g and 0.37 pg/g, respectively. For the South region, the highest value of the total PCBs was obtained at 0.64 pg/g for *Scomberomorus guttatus* (Spanish mackerel), followed by 0.54 pg/g in *Chirocentrus dorab* (Dorab wolfherring) and 0.35 pg/g for *Megalapsis cordyla* (Hardtail scad). In the Middle region, it was shown that the highest level of total PCBs was detected in *Megalapsis cordyla* (Hardtail scad), with the value of 1.05 pg/g wet weight, followed by *Eleutheronema tradactylum* (Fourfinger threadfin) and *Rastrelliger kanagurta* (Indian mackerel), with the values of 0.47pg/g and 0.25pg/g, respectively. In Table 2, different levels of the total PCBs can be present in the same species, depending on the sizes, maturity and total fat of the fish. When comparing the regions in the first trip of sample collection, those from the North region were found to contain the highest level of PCBs. The North region (comprising of Penang, Kedah and Perlis states) is within the vicinity of famous tourism place (i.e. Langkawi Island) which is close to one of the biggest ports in Kuala Perlis and neighbouring with plastics and agricultural industries in the north Peninsular. It has been well established that aquatic organisms situated within human and industrial activities may bio-accumulate chlorinated organic compounds in their bodies (Smeds & Saukko, 2001; Ntow, 2001).

In the second trip of sample collection, the Middle region showed the highest concentrations of PCBs in three pelagic species. In particular, *Megalapsis cordyla* (Hardtail scad) demonstrated the highest level of the total PCBs (0.77 pg/g wet weight), compared to *Eleutheronema tradactylum* (Fourfinger threadfin) and *Rastrelliger kanagurta* (Indian mackerel), which were at 0.38 pg/g and 0.35 pg/g wet weight, respectively. In the South region, on the other hand, *Scomberomorus guttatus* (Spanish mackerel) contained the highest total PCBs at 0.72 pg/g of wet weight, whereas *Chirocentrus dorab* (Dorab wolfherring) and *Megalapsis cordyla* (Hardtail scad) had about 0.68pg/g and 0.50pg/g of wet weight, respectively. For the samples taken from the North region, *Eleutheronema tradactylum* (Hardtail scad) and *Pampus argenteus* (Silver pompret) showed the highest values of the total PCBs concentration at 0.55 pg/g wet weight and 0.53 pg/g wet weight, respectively. Meanwhile, *Rastrelliger kanagurta* (Indian mackerel) and *Megalapsis cordyla* (Hardtail scad) had lower concentrations of the total PCBs at 0.35 pg/g wet weight in both species. A study by Bocio *et al.* (2007) in Spain showed that the mackerel species contained the PCBs level of 0.89 pg/g of wet weight, which is within the range (0.35-1.37 pg/g of wet weight) of the values obtained in the present study. Different species of mackerel (*Scomber scombrus*) from the Adriatic sea in Italy was reported to contain PCBs at 3015 pg/g wet weight, which is much higher than the value obtained in the present study (Storelli *et al.*, 2003). The findings of this study have clearly shown that the level of PCBs varies greatly between Trip 1 and 2 as variation, i.e. ranging from 0 to 83.87% across the different species and regions. The differences in the level of PCBs in the fish samples are dependent upon the surrounding human activities and industrial waste releases. In all the studied samples, PCBs were found to be generally presence in all the samples at varied concentrations. However, the levels were well below the permitted level of

TABLE 3
Polychlorinated biphenyls (four non-*ortho*-PCBs and eight mono-*ortho*-PCBs congeners) and toxic equivalents (WHO-iTEQ) as pg/g in the fish species

Fish Analytes	Hardtail scad	Fourfinger threadfin	Indian mackerel	Silver pompret	Dorab wolfherring	Spanish mackerel
Non-*ortho* PCBs	1.0	0.44	1.29	0.46	0.62	0.68
Mono-*ortho* PCBs	0.05	0.11	0.08	0.07	0.06	0.04
WHO-iTEQ (pg/g)	1.05	0.55	1.37	0.53	0.68	0.72

Non-*ortho* PCBs include (PCBs 77, 81, 126 and 169)
Mono-*ortho* PCBs include (PCBs 105, 114, 118, 123, 156, 157, 167 and 189)

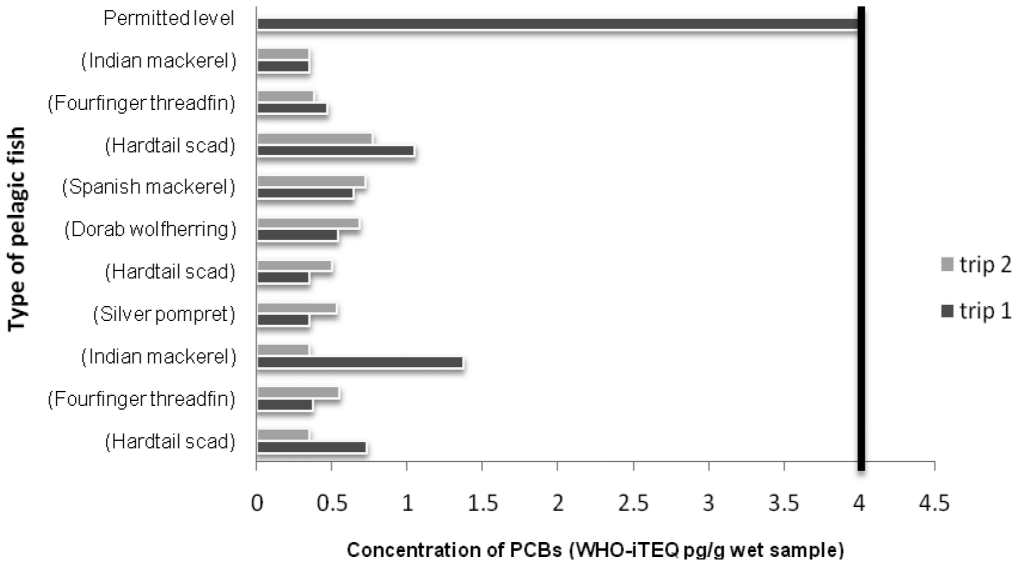
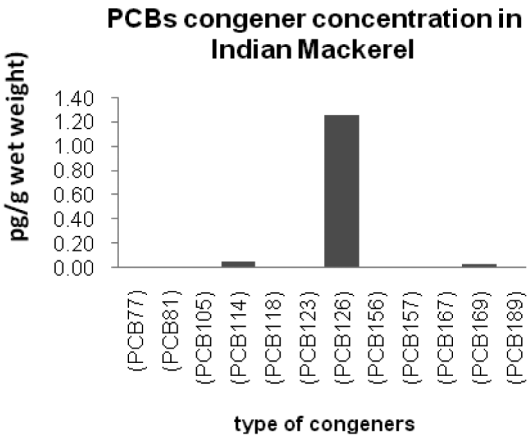
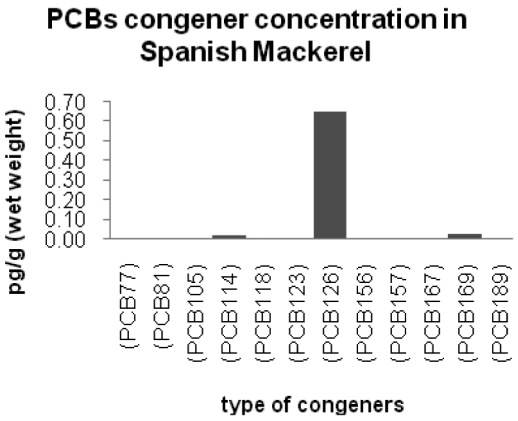


Fig. 2: Total PCBs in the pelagic species (WHO-iTEQ pg/g wet weight)

4 pg/g for muscle fish and fishery products, as shown in Figure 2 (Van den Berg *et al*., 2006).

Table 3 shows congeners of all the samples that were further divided into non-*ortho* (PCBs 77, 81, 126 and 156) and mono-*ortho* (PCBs 105, 114, 118, 123, 156, 157, 167 and 189) families. There were more than 80% of the non-*ortho* PCBs which dominated in the samples compared to the mono-*ortho* congeners. The profile of polychlorinated congeners in the fish samples caught from the Straits of Malacca was distinct from those taken from the southern areas of the Adriatic Sea (Storelli *et al*., 2003), the Mediterranean waters of Tunisia (Masmoudi *et al*., 2007) and farmed Rainbow Trouts in Southern Finland (Kiviranta *et al*., 2001). Based on the findings of the present study, the Indian mackerel attained the highest amount of non-*ortho* (1.29 pg/g) and mono-*ortho* (0.08 pg/g) PCBs. Meanwhile, the lowest amount of non-*ortho* and mono-*ortho* was observed in Silver pompret, at 0.46pg/g and 0.07 pg/g, respectively. A study by Fernandez *et al*. (2004) revealed that Mackerel species (known as *Scomber scombrus*) contained 1.58 pg/g and 1.95 pg/g of non-*ortho* and mono-*ortho* PCBs,





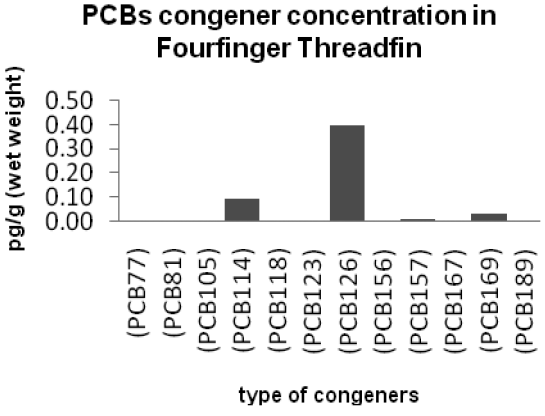


Fig. 3: Type of the pelagic fish with fat contents >2%

respectively. These samples were collected in Spain and the amount was much higher than the levels of non-*ortho* (0.68 pg/g) and mono-*ortho* (0.04 pg/g) of *Scomberomorus guttatus* (Spanish mackerel) obtained in the present study. These discrepancies may due to the differences in the geographical area, size, maturity and species of the fish although they came from the same family.

Besides determining the total PCBs concentration in the fish species, this study was also looking at the amount of each congener of PCBs. All of the species undertaken in this study had their own congener profile. According to Bocio *et al*. (2007), the predominant PCB congeners are PCB 118 and PCB 126, with PCB 126 being the most toxic (WHO-TEF = 0.1) to human, and can be accounted for the largest contribution of PCBs to the TEQs for each species (Fernandez, *et al*., 2004). PCB 126 was present in most of the samples studied in the present study. In fact, PCB 126 was found to be highest in Indian mackerel (1.26 pg/g) but lowest in Silver Pompret (0.30 pg/g). Other congeners like PCB 114 and PCB 169 were also found in most samples, including Hardtail scad, Indian Mackerel, Spanish Mackerel and Fourfinger threadfin (Figure 3), but in low concentrations.

CONCLUSION

This study has identified the total PCBs concentration, including the type and level of 12 congeners of PCBs that are most toxic to humans. The most toxic congener, i.e. PCB 126, was detected in the samples at relatively low concentration in the muscle tissue of fish species collected along the Strait of Malacca. Nonetheless, the values obtained for the total PCBs in this study were generally well below the permitted level recommended by WHO. Therefore, it can be concluded that the pelagic fish taken from the Strait of Malacca are safe to consume, based on their PCBs level. Although the level was not high, it is still important to set limits for the PCB levels in fish species to have a better estimation of the risk of exposure to human through dietary intake of fish, specifically fatty fish, to meet nutritional requirement for long-chain n-3 polyunsaturated fatty acids.

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Status and Distribution of Non-volant Small Mammals in Universiti Putra Malaysia, Bintulu Sarawak Campus (UPMKB)

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ABSTRACT

This paper presents basic distribution data on non-volant small mammals derived from four sampling periods (2006-2009) in UPMKB. A total of 22 species belonging to families Tupaiidae, Sciuridae, Muridae, Viverridae, Manidae, Tarsiidae, Felidae and Cercopithecidae were captured and recorded. Planted Forest showed the highest number of species (14), followed by Nirwana Forest (11 species), Campus Zone Area (10 species), and Forestry Park (5 species). Two (2) species have been classified as Totally Protected while seven (7) species are protected under the Sarawak Wildlife Protection Ordinance, 1998. Mapping of the total area showed that the Campus Zone Area contained the highest diversity and the number of non-volant small mammals as compared to other sites in UPMKB. Thus, conservation effort of the area should be given higher priority to ensure that the biodiversity of the area is maintained.

Keywords: Distribution, mapping, non-volant small mammals, totally protected species, protected species

INTRODUCTION

The distribution of mammals, especially endangered species, has been used to justify the establishment of new conservation units (Bonvicino *et al.,* 2002). In Borneo, primates have received most attention. Several Bornean conservation units in Sarawak, such as Lanjak-Entimau Wildlife Sanctuary and Batang Ai National Park, have been established to protect the endangered primate species. Full attention given on selected flagship mammals, such as tiger, rhino and elephant, resulted in neglecting much less known species, like non-volant small mammals (weight of < 5kg) which occur in complex communities with exceptional high species richness (Zubaid & Ariffin, 1998; Bernard, 2004; Wells *et al.,* 2004).

On the contrary, research performed on the habitat use by non-volant small mammals in Malaysia and in Borneo is still very limited. This is because the species, which are highly cryptic in their appearance and behaviour, hardly visible to the human observers, and this is also hindered by the dense and evergreen vegetation, apart from the fact that some species are active at night, hiding in burrows or tree holes during the day (Wells *et al.,* 2004).

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Beginner level of research in conserving habitat for non-volant small mammals within the university/campus areas was conducted at Universiti Putra Malaysia Bintulu Sarawak Campus (UPMKB).

The objective of this research was to explore the diversity, distribution and status of the species as UPMKB consisted of lush environment that is rich in flora and fauna. Out of the green area covering about 299.0 ha (42%), 37.3 ha was assigned for Rehabilitation of Tropical Rainforest Ecosystem Project in Sarawak, *Gmelina arborea* and *Acacia mangium plantations* (25.5 ha), oil palm plantation (51 ha), rubber estate (25 ha), Nirwana Forest (secondary forest) (60 ha) and fragments of the secondary forest (100.2 ha). The rest of the area (416.16 ha) consists of the existing and future development areas (Ong *et al.,* 2008).

Due to the large green area, less disturbed and abundance of resources such as foods and nesting areas, it is believed that UPMKB can play an important role as a green lung for Bintulu area. Therefore, determining the diversity, distribution and status of the species could help in the future implementation of proper management and conservation strategies in this area.

MATERIALS AND METHODS

The study sites include Campus Zone Area (CZA), Planted Forest (PF), Nirwana Forest (NF) and Forestry Park (FP). CZA comprised of agricultural area, plantation area, animal farm, mechanization workshop, administration and academic area, Botanical Park, as well as Biopark and waterfall area. Planted Forest covers an area of 3.4 ha, with more than 352,694 indigenous forest trees from 127 species on five planting sites with a total of 119 plots set-up (Mohamad Azani *et al.,* 2003). The Nirwana Forest is a secondary forest that is mostly dominated by species from the family, *Dipterocarpaceae*. Meanwhile, the Forest Park area covers about 40 ha of land and is considered as a lowland secondary forest.

The small non-volant mammals in all study sites were trapped using different sizes (small, medium and large) of live traps that were baited alternately with banana, coconut, jack fruit, peanut butter and chicken parts. The traps were randomly set up on the forest floor, fallen logs and branches of trees from early 2006 until January 2009. The traps were checked between 0700-0900 hours in the morning and 1700-1800 hours in the afternoon. Then , the traps were rebaited and set back at the same locations. All the captured animals were identified, weighed and measured according to the procedure by Payne *et al*. (1985) to aid positive identification. Two camera traps (namely, Digital Ranger S600 CB, and Cam Trak South, Inc., US) were set at 1.5 m above the ground during the study.

All the original sites where the animals were captured were revisited and geographically positioned using Garmin GPS Handheld receiver. In determining the distribution area of non-volant small mammals in UPMKB, their recorded data were geo-referenced and registered in ArcView GIS. Meanwhile, maps showing the locations of the occurrences of non-volant small mammals in that area were also established.

RESULTS AND DISCUSSION

A total of 152 non-volant small mammals were recorded during this study period. At the same time, eight (8) families comprising of 22 species were identified, with most of the animals caught using live traps (Table 1). The two most frequently caught species were *Callosciurus notatus* and *Rattus tiomanicus jolarensis* which accounted for 24.34% and 15.80% of all the captures, respectively. The existence of *Felis bengalensis and Macaca nemestrina* was recorded via camera trapping. In addition to this, three families (*Manidae, Tarsiidae,* and *Viverridae*) and three species (*Manis javanica, Tarsius bancanus* and *Viverra tangalunga*) were added to the diversity list through the observation in PF area. Eight individuals of *T. bancanus* were captured in the NF area. The animals were found trapped in the mist nets

TABLE 1
The list of non-volant small mammal's occurring at four study sites during four sampling periods from 2006 to 2009. (Conservation status: TP= Totally Protected; P= Protected; NP= Not Protected; based on Sarawak Wildlife Protection Ordinance, 1998)

No.	FAMILY/*Species*/Local Name	Conservation Status	N	Abundance			
				CZA	PF	NF	FP
	TARSIIDAE						
1	*Tarsius bancanus* (Western tarsier)	TP	9	0	1	8	0
	SCUIRIDAE						
2	*Ratufa affinis* (Giant squirrel)	TP	2	2	0	0	0
3	*Callosciurus notatus* (Plantain squirrel)	NP	37	28	2	6	1
4	*Callosciurus prevostii caroli* (Prevost's squirrel)	NP	4	2	1	0	1
5	*Callosciurus adamsi* (Ear-spot squirrel)	NP	3	0	3	0	0
6	*Rhinosciurus laticaudatus* (Shrew-faced ground squirrel)	NP	5	1	2	2	0
	TUPAIIDAE						
7	*Tupaia gracilis* (Slender treeshrew)	P	1	1	0	0	0
8	*Tupaia minor* (Lesser treeshrew)	P	5	3	0	2	0
9	*Tupaia tana* (Large treeshrew)	P	16	5	2	1	8
	MURIDAE						
10	*Sundamys muelleri* (Muller rat)	NP	12	0	9	3	0
11	*Rattus tiomanicus jolarensis* (Malaysian field rat)	NP	24	23	0	1	0
12	*Rattus argentiventer* (Rice field rat)	NP	1	0	0	1	0
13	*Rattus baluensis* (Summit rat)	NP	9	7	2	0	0
14	*Maxomys alticola* (Mountain spiny rat)	NP	2	0	1	0	1
15	*Maxomys rajah* (Brown spiny rat)	NP	8	0	0	8	0
16	*Rattus rattus* (House rat)	NP	8	6	1	1	0
17	*Niviventer rapit* (Longtail mountain rat)	NP	1	0	1	0	0
18	*Sundamys infraluteus* (Mountain giant rat)	NP	1	0	0	0	1
	FELIDAE						
19	*Felis bengalensis* (Leopard cat)	P	1	0	1	0	0
	VIVERRIDAE						
20	*Viverra tangalunga* (Malay civet)	P	1	0	1	0	0
	MANIDAE						
21	*Manis javanica* (Pangolin)	P	1	0	1	0	0
	CERCOPITHECIDAE						
22	*Macaca nemestrina* (Pig-tailed macaque)	P	1	0	0	1	0
	Total species, s		22	10	14	11	5
	Total individual, *N*		152	78	28	34	12

which were used to capture bats. Giant squirrel, *Ratufa affinis*, was only recorded in the CZA through the observation.

From the results, fourteen species of non-volant small mammals were recorded in the PF area, followed by the NF area (11 species), CZA (10 species) and five species were observed in the FP area. In term of diversity indices (Table 2), the results indicated that the total of species was quite different in all the study sites based on the richness index R1. As for species evenness, the distribution of the individuals among the species was shown to be uneven in FP (E=0.69), CZA (E=0.72) and PF (E=0.86) compared to NF (E=0.94). Based on Hill's diversity index, the number of abundant (N_1) and very abundant species (N_2) was respectively higher in PF compared to the other sites. Meanwhile, the Shannon-Weaner diversity index further indicated that species composition was higher in PF (H=2.30), followed by NF (H=2.05), CZA (H=1.74) and FP (H=1.1). This result is similar to that of Wells *et al.* (2006) who found that the diversity of small mammals was significantly reduced in secondary forest with common species recorded in all the study sites.

TABLE 2
Diversity indices of the species of non-volant small mammal at four study sites; Campus Zone Area (CZA), Planted Forest (PF), Nirwana Forest (NF) and Forestry Park (FP)

Indices	Study sites			
	CZA	PF	NF	FP
Species Richness				
No	10	14	11	5
R1	2.07	3.9	2.84	1.61
Species Diversity				
H'	1.74	2.31	2.05	1.10
N_1	5.71	10.08	7.79	3.00
N_2	4.40	8.79	7.38	2.36
Species Evenness				
E	0.72	0.86	0.94	0.68

No = Total number of species, R1 = Margalef index; H = Shannon index N1 = Measures the number of abundant species present; N_2 = Measures the number of very abundant species; E = Evenness indices

Approximately 8.7% (2 species) of the total non-volant small mammals recorded in this study are totally protected, 30.4% (7 species) are protected, while the remaining are listed in the non-protected status under Sarawak Wildlife Protection Ordinance, 1998.

One of the totally protected species, *T. bancanus,* was captured in NF as well as observed in PF. This species is known as arboreal which uses trees as feeding or sleeping sites (Yustian *et al.*, 2008); it was also found in higher estimated population density in forest with small-scale interspersed plantation (Merker & Muehlenberg, 2000). Therefore, NF and PF can be suggested as suitable places for their habitat as these forests are dominated by a variety of tall tree species with small scale interspersed trees that can facilitate their foraging and sleeping activities. Thus, the two sites are important as tarsier conservation areas in UPMKB.

The other totally protected species, i.e. *Ratufa affinis*, was only recorded in Botanical Garden in CZA. According to Payne *et al.* (1985), *R. affinis* is active in tall trees and descending to the ground only. In the observation carried out in this study, *R. affinis* was found foraging and nesting at the top of big and tall *Acacia mangium* tree in the Botanical Garden as it adapted to the fragmented forest which is buffered by lower secondary forest (Forestry Park) nearby.

Felis bengalensis, Viverra tangalunga and *Manis javanica* confirmed present in PF through photographic results of camera trapping and via direct observation. The presence of these species in PF reflects their ecological and habitat adaptation. *F. bengalensis* usually occur in forest and gardens and can also be present in plantations, as they are agile and elusive and have adapted well to their environment (Krieger, 2007). Meanwhile, *V. tangalunga* can occur in the forest and cultivated lands which are adjacent to the forest. *M. javanica*, on the other hand, always recorded the presence in tall and secondary forests, as well as from the cultivated area including gardens (Payne *et al.,* 1985).

Unlike Plantain squirrel, Ear-spot squirrel (*Callosciurus adamsi*) can only be found in PF.

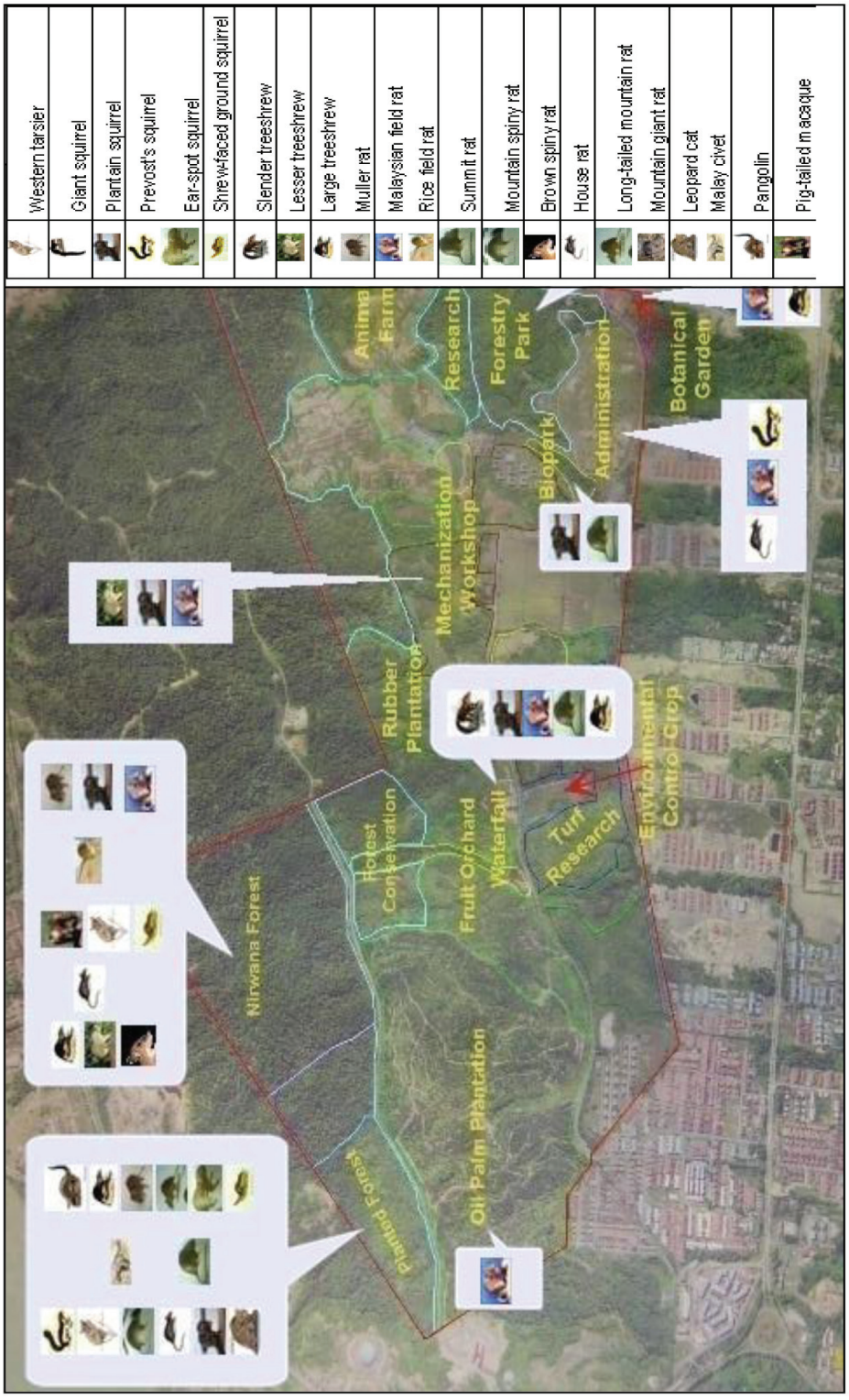

Fig.1: A mapping distribution of non-volant small mammal species in UPMKB; Campus Zone Area, Planted Forest, Nirwana Forest and Forestry Park

This species is particularly active in small trees (Payne *et al.,* 1985) and some part of PF consists of this type of habitat. Meanwhile, *Tupaia gracilis* was recorded in the forest area, as well as gardens and plantations (Payne *et al.,* 1985), suggesting that this particular species could adapt to the disturbed habitats such as CZA. *Rattus argentiventer* and *Maxomys rajah* were trapped in NF. According to Nowak (1991), *R. argentiventer* resides in cultivated areas, such as rice field and grasslands and they are dependent on rice fields and plantation. The presence of this species at NF may be due to the habitat edge effect as NF is located near oil palm plantation. Meanwhile, *M. rajah* occurs predominantly in tall secondary forest and their diets are almost identical (Payne *et al*., 1985; Wells *et al.,* 2006). *Niviventer rapit* has been found in PF as this species is active in small trees (Payne *et al.,* 1985).

Pig tailed macaque, known as *Macaca nemestrina,* was recorded by the infrared censored camera in NF and this particular species was commonly encountered near the forest fringes and while crossing the road to the oil palm plantation during the day. This species is usually found in hilly forests, and they sometimes enter plantations or gardens that are located adjacent to lowlands forest where they cause considerable damages to grain and fruit crops (Payne *et al.,* 1985). This result is also supported by the finding of an earlier study which suggested that *M. nemestrina* spends substantial time on the ground compared to the species of other primates (Mohd Azlan, 2006).

CONCLUSIONS

Mapping distribution of non-volant small mammals has revealed that UPMKB has a potential to be used as a suitable buffer zone; this is particularly because as the planted forest, the rehabilitated forest has been found to be rich in species diversity and all the hot spots highlighted on the map should be monitored, protected and managed according to the development plans set for the campus development.

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Over-expression of *Escherichia coli* Transaldolase in the Cytosol of *Arabidopsis thaliana*

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ABSTRACT

Transaldolase (TAL) is an enzyme of the oxidative pentose phosphate pathway (OPPP) which catalyzes the reversible reaction of sedoheptulose-7-phophate into fructose-6-phosphate and erythrose-4-phosphate. In some micro-organisms, fungi and plants, erythrose-4-phosphate condenses with phosphoenolpyruvate (PEP) from glycolysis to form chorismate which is a precursor for many secondary metabolic pathways such as aromatic amino acids, flavonoids, lignin, indole acetate and UV light protectants. An analysis of plant genome databases reveals that the OPPP is incomplete in the cytosol of plants as no genes encoding for a cytosolic transaldolase (TAL) and transketolase (TK) have been identified so far. Thus, this study attempted to complete the compartmentation of TAL in the cytosol and plastid of plants by over-expressing it in the cytosol of *A. thaliana*. For this purpose, homozygous transgenic plants were obtained in these studies; it was found that the transaldolase activity of transgenic lines increased as compared to wild type plants. The findings of the current study also demonstrated that transgenic plants did not show any distinct phenotypes and there was no difference in a range of growth parameters compared with *A. thaliana* Col-0 (wild type).

Keywords: ***Agrobacterium tumefaciens,*** **transaldolase, oxidative pentose phosphate pathway, transgenic plants, shikimate pathway**

INTRODUCTION

Oxidative Pentose Phosphate Pathway (OPPP) is involved in the metabolism of carbohydrates *via* the oxidation of glucose-6-phosphate. The pathway is composed of two phases, namely, the oxidative and the non-oxidative. The first phase is irreversible and it consists of the oxidation of glucose-6-phosphate that leads to the production of ribulose-5-phosphate. A large percentage (50-60%) of the required NADPH, a major reducing power for various anabolic pathways including the biosynthesis of fatty acids, the reduction of nitrite (Sprenger, 1995; Dennis *et al.*, 1997; Debnam *et al.*, 2004), for cell protection against oxidative stress and synthesis of glutamate (Schnarrenberger *et al.*, 1995) is contributed by the first phase. The second phase consists of a reversible series of interconversion between 3-, 4-, 5-, 6- and 7-carbons sugar that are catalyzed by the enzymes ribose-5-phosphate isomerase (RPI), ribulose-5-phosphate-3-epimerase (RPE) and transketolase (TK) and transaldolase (TAL).

In some micro-organisms, fungi and plants, erythrose-4-phosphate condenses with phosphoenolpyruvate (PEP) from glycolysis to form chorismate, i.e. the first substrate for the shikimic pathway which leads to the production of aromatic amino acids and many aromatic

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secondary metabolites, such as flavonoids, indole acetate, UV light protectants and lignin (Hermann & Weaver, 1999). These metabolites play an important role in the interaction of plant with the environment. However, its contribution to carbon metabolism in green plants is difficult to assess as plants have dual compartmentations.

The OPPP operates in the cytoplasm of bacteria, cyanobacteria, yeast and animals. Many studies have focused on the determination of the subcellular compartmentation of the OPPP enzymes in plants, such as measurement of the enzymatic activities, and measurement of the fluxes through the OPPP enzymes by feeding with radio-labelled substrates; however, the results are still controversial and uncertain due to the limitations of each technique. Several database networks (e.g. www.genome.jp/kegg/genes.html; www.tigr.org; http://mips.gsf.de; www.arabidopsis.org and www.ncbi.nlm.nih/gov) reveal that most the OPPP enzymes are present in both the cytoplasm and the plastid, except possibly TAL and TK, which are thought to be plastid localized. The latter would result in an incomplete OPPP in the cytosol of the plant cell. Thus, it can be concluded that there is no genetic evidence for a complete OPPP in the cytosol of plant to date. The aim of this study was to over-express *E. coli TAL* in the cytosol of *A. thaliana* Col-0 in order to produce transgenic plants which have a complete OPPP in the cytosol. Two genes encoding distinct isoforms of *E. coli* TAL, *talA* (GenBank accession number P0A867) and *talB* gene (GenBank accession number P0A870) were used in this study to over-express in *A. thaliana*.

MATERIALS AND METHODS

Preparation of the Plant Material for Transformation

A. thaliana ecotype Columbia (Col-0) was grown in the controlled-environment growth cabinet in the Central Annexe Facilities, University of Sheffield. The growth cabinet was set at 20°C and an irradiance of photosynthetic photon flux density (PPFD) of 250 $\mu molm^{-2}s^{-1}$. Meanwhile, photoperiod was set for 14 hours for transformation and seed production.

Constructions of E. coli *TAL to Over-express in Cytosol*

E. coli talA and *talB* (obtained from Dr. Sachiko, University of Sheffield) were ligated in the sense orientation into the *XbaI* and *SstI* sites of the pMOG22. The ligations were then sub-cloned into subcloning efficiency DH5α competent cells (Gibco). The transformed *E. coli* were spread on the LB (Luria-Bertani) plates (5.0 g NaCl, 10.0 g Bacto Tryptone, 5.0 g Bacto Yeast Extract, 15 g of Bacto-Agar, 100 µl of 10 N NaOH and ddH_20) containing antibiotic kanamycin (50 mg/L). Successful transformants were identified by the formation of colonies on the media. The plasmid vectors were purified from positive colonies by minipreps and the isolated plasmids were further analysed by agarose gel electrophoresis.

Construction of Agrobacterium *Electro-competent Cells*

Agrobacterium tumefaciens (C58) was grown in 50 ml of YMB medium (0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.1 g L^{-1} NaCl, 10 g L^{-1} mannitol, 0.4 g L^{-1} yeast extract and pH 7.0, HCl) with 200 mg L^{-1} rifampicin for 2 days at 28ºC with shaking. The culture was kept cooled on ice for 30 min and then centrifuged at 4,000×g at 4ºC for 10 min. The pellet was resuspended in 1 ml of 10% (v/v) glycerol.

Electroporation of Agrobacterium

E. coli TAL constructs were inserted into *Agrobacterium* through electroporation, in which 100 µl of *A. tumefaciens* and 2 µl of each constructs were electroporated by using Bio-Rad GenePulser (Miller & Nickoloff, 1995).

Arabidopsis Transformation by *Agrobacterium* Mediated Method

Preparation of the Agrobacterium Cultures

Agrobacterium tumefaciens, C58, was grown in 10 ml YMB cultures with 50 mg/L hygromycin at 28°C for 2 days. 1 ml of the cultures was inoculated into 250 ml of YMB cultures with 50 mg/L hygromycin and incubated at 28°C for 2 days. The cultures were then centrifuged at 3,000 x g for 10 min at 4°C, and the pellets were resuspended in 5% sucrose to give the OD at 600nm, OD_{600}=0.8-1.5 before 0.05% Silwet was finally added prior to dipping plants.

Transformation

Arabidopsis was transformed by immersion of inflorescences in a suspension of *A. tumefaciens* solution for 5-10 seconds with gentle agitation (Clough & Bent, 1998). The plants were then covered with black plastic overnight. It is important to note that these plants were watered as usual and this was stopped once the seeds became mature. All self-fertilized seeds were collected to screen transgenic lines.

Screening of the Transgenic Lines

Sterilisation of Seeds and Selection of Hygromycin-Resistant Transformants

Seeds were sterilized with 50% (v/v) ethanol and left at room temperature for 10 min prior to centrifugation at 10,000 rpm for 30 sec. The seeds were then washed with 50% (v/v) bleach and vortex vigorously, left for 5-10 min at room temperature and centrifuged at 10,000 rpm for 30 sec. These seeds were then rinsed four times with sterile distilled water and dried under sterile condition. Later, the sterilized seeds were plated onto the MS media with 200 mg/L hygromycin and grown for 2 weeks in the growth chamber (Versatile Environmental Test Chamber, Sanyo) at 20°C in the light (200 µmol quanta $m^{-2}s^{-1}$) with 10 hours of photoperiod.

Small Scale DNA Extraction from Plant Material for the PCR Analysis

Small pieces of leaf samples were taken from the plants at week 3. Pre-heated (65°C) DNA extraction buffer (200 mM Tris, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and ground using a mixer mill for 5 min. The samples were incubated in the water bath at 65°C for 15 min, followed by centrifuging at 14,000 rpm for 10 min. Supernatant was transferred into a 1.5 ml tube containing isopropanol with 1:1 ratio and mixed by inverting the tube several times. The DNA was precipitated at room temperature for 15 min. The samples were then centrifuged at 14,000 rpm for 15 min and the supernatant was discarded. The pellet was washed twice with 50 µl of 70% ethanol and air-dried. Finally, the pellet was resuspended in 50µl of sterile distilled water and stored at -20°C until subsequent molecular analysis.

Measurement of the Transaldolase Activity

Supernatant (as prepared above) was desalted with PD10 column. A solution (1ml) containing 50 mM HEPES-HCl, pH 8.5, 20 mM NADH, 20 mM F6P and 0.6U/1.8U glycerol 3-phosphate dehydrogenase/triose phosphate isomerase enzyme mixture (G3PDH/TPI) and desalted supernatant was measured spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, UK) at 340 nm to quantify transaldolase activity (Brand, 1983; Schnarrenberger *et al*., 1995; Sprenger *et al.*, 1995).

Immunoblotting

Preparation of the Samples

Sample buffer (62.5 mM Tris-HCl, pH 6.8, 20 % [v/v] glycerol, 10 % SDS, 5 % [v/v] β-mercaptoethanol, 0.012 % [v/v] bromophenol blue) was added to the supernatant (as prepared above) in the ratio of 1:1 ratio before it was boiled for 5 min.

Preparation of Gels and Electrophoresis

Proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) by using Mini-Protean 3 cell (Bio-Rad). The gels consist of upper 5 % stacking gel (5.7 ml ddH_2O, 1.7 ml 30% degassed Acrylamide/Bis, 2.5 ml gel buffer 0.5 M Tris-HCl pH 6.8, 100 µl of freshly prepared 10 % (w/v) SDS, 50 µl 10% (w/v) APS and 10 µl NNN'N'-tetramethylethylenediamine (TEMED) and lower 10% running/resolving gel (3.4 ml ddH2O, 4.0 ml 30% degassed Acrylamide/Bis, 2.5 ml gel buffer 1.5 M Tris-HCl pH 8.8, 100 µl 10% (w/v) SDS, 50 µl of the freshly prepared 10 % (w/v) APS and 5 µl TEMED).

Immunoblotting

Protein samples (15 µg) were loaded on the gel and run at 200 V for an hour (Bio-Rad, USA). The proteins were then transferred to Immobilon-P polyvinylidene difluoride membranes (PDVF) (Millipore, Bedford, UK) at 30 V for overnight membranes using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), according to the manufacturer's instruction. After the transfer, the membranes were washed in TBS (20 mM Tris-HCl, pH7.4, 140mM NaCl) by shaking for them for 2-5 min at room temperature. The membranes were blocked with 3% milk/TBS for 1 hour on the orbital shaker and washed four times with TBS for 5 min. The membranes were then probed with *talA* or *talB* antibodies (obtained from Dr. Sachiko, University of Sheffield) for 1 hour on the orbital shaker. The primary antibodies were prepared as a 1:1000 dilution in 3% milk/TBS. The membranes were washed four times with TBS for 5 min and subsequently probed with secondary antibodies for 1 hour on the orbital shaker, after which time they were washed with TBS for 5 min, and this washing step was repeated four times. The secondary antibodies conjugated with peroxidase were prepared as a 1:1000 dilution in 3% milk/TBS. Finally, the protein bands were visualized using an ECL chemiluminescence detection reagent kit (Amersham Life Sciences Limited, Buckinghamshire, UK). The band image was captured on Kodak BioMax MR film (Kodak, New York, USA).

RESULTS AND DISCUSSION

Construction of the Plant Transformation Vectors

This study used prokaryotic TAL from *E. coli*, encoded by *talA* and *talB* genes (obtained from Dr. Sachiko Shimizu, University of Sheffield) in order to avoid gene silencing of the endogenous gene. The introduction of a homologous gene expressed constitutively in the plant cell can often result in the silencing of the endogenous gene via co-suppression (Primrose *et al.*, 1996). Therefore, *E. coli* TAL is suitable to be over-expressed in the plants because it has low homology to plant genes of about 43% to 49% (Caillau, 2002). The suitability of *E. coli* TAL to be over-expressed in plants had been proven by Shimizu (2002), based on the kinetic properties of *E. coli TAL*, which has an optimum pH 8.5-9.5, while the optimum pH of recombinant transaldolase of tomato is 8.3-8.8 (Caillau, 2002). Thus, *E. coli* TAL will be active when they are over-expressed in plants because they have similarities in pH optima. The Km value of F6P and E4P of *E. coli* TAL are 1.2 mM and 0.09 mM, while the Km value of F6P and E4P of spinach are 1.0 mM and 0.05 mM (Tsolas & Horecker, 1972*)* and the recombinant transaldolase of tomato are 0.35 mM and 0.13 mM (Caillau, 2002). Therefore, a range of the Km value of F6P and E4P of *E. coli TAL* and plant TAL is similar, which is 0.35 to 1.20 mM for F6P and this is 0.05 to 0.13 mM for E4P. Thus, it is assumed that the concentrations of F6P and E4P are high enough to drive the maximum activity of TAL in the cytosol.

Plant transformation vectors were constructed for the expression of *E. coli* TAL in the cytosol of *A. thaliana*. *E. coli talA* and *talB* were ligated in the sense orientation into the *XbaI* and *SstI* sites of the pMOG22 backbone (*Fig. 1a*). Figure 1b shows that the construction

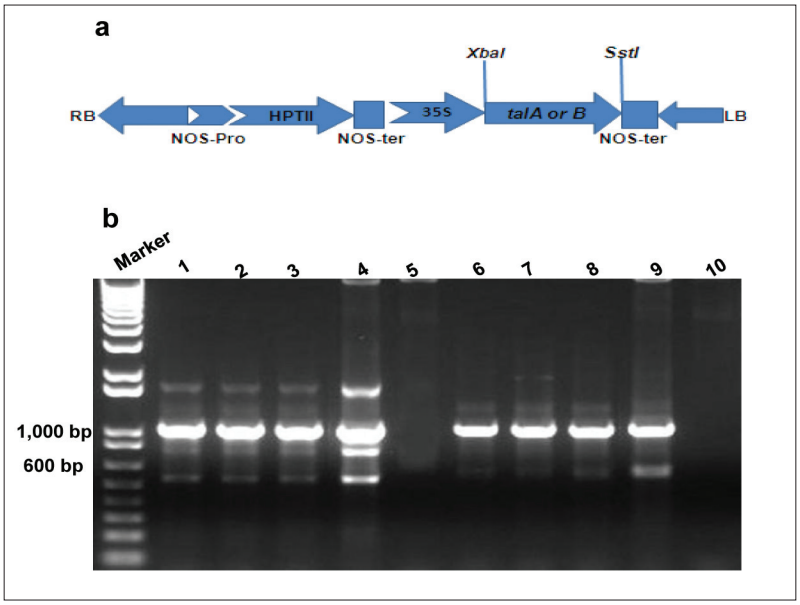

*Fig. 1: The structure of plant transformation vector pMOG22-35S-*talA *or* talB *and PCR analysis of pMOG22-35S-*talA *and* talB. *a) T-DNA region containing the Hygromycin gene (HPTII) as a selectable marker under the control of the nopaline synthase promoter (NOS-pro), and followed by NOS-ter.* E. coli talA *or* talB *were inserted in the sense orientation between XbaI and SstI restriction sites. The expression of both TAL genes is driven by the CaMV 35S promoter. RB: right border; LB: left border of T-DNA. b) clones were subjected to PCR to ensure that ligation reaction was successful. Lanes 1-4 show bacterial colonies containing* talA *gene. Lanes 6-9 show bacterial colonies containing* talB *gene. Lanes 5 and 10: negative control and empty pMOG22 binary vector*

of plant transformation vectors was successful. Then, the constructs of *talA* and *talB* plasmids were electroporated into *Agrobacterium* electro-competent cells.

Introduction of E. coli *TAL into* A. thaliana *via* Agrobacterium*-mediated Transformation Method*

Fig. 2a shows that transgenic plants were clearly identified as they produced green and healthy secondary leaves. Roots deeply invaded into a medium containing antibiotic hygromycin. Polymerase Chain Reaction (PCR) analysis showed that the putative transgenic plants could be identified by specific amplification of a 600 bp product corresponding to the *E. coli* TAL on the agarose gel (*Fig. 2b*).

The expression of *E. coli* TAL in *Arabidopsis* was further analyzed by immunoblotting and measurement of transaldolase activity. The protein extract from the transgenic plants showed a strong immuno-reaction with antibodies against *talA* or *talB*. The transaldolase activity of transgenic plants is about 2 to 6-folds higher than that of the wild type plants. These results also showed that the levels of expression of *E. coli talA* and *talB* varied among the transgenic plants (Figure 3a). Immunoblot band intensity of the transgenic plants was found to be positively correlated with transaldolase activity, which is *talA* transgenic lines ($r=0.732$, $p<0.01$) and in *talB* transgenic lines ($r=0.975$, $p<0.01$). This indicated that both over-expressed of TAL protein were functional in transgenic lines.

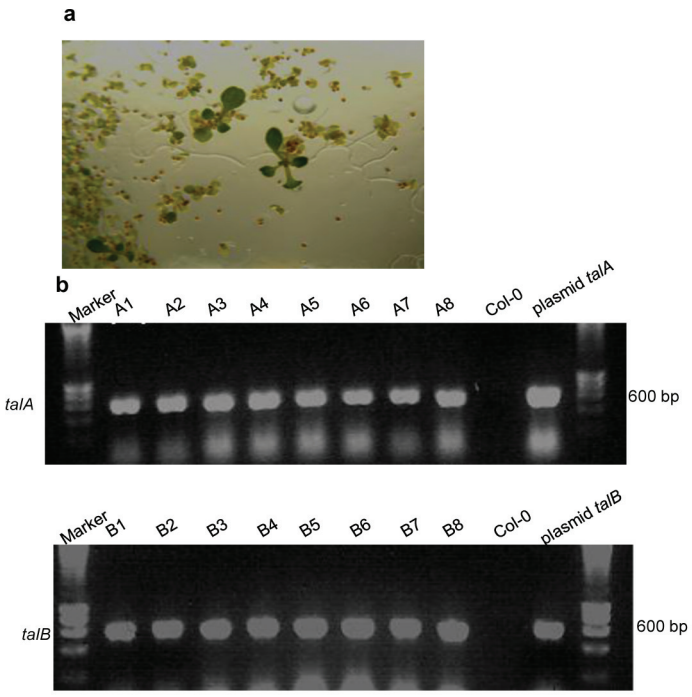

Fig. 2: Screening of transgenic Arabidopsis *plants. a, Seedlings showing resistance to hygromycin were identified as transgenic plants. b) PCR screening of transgenic plants. Col-0:*A. thaliana *Col-0 (wild type)*

Before carrying any further analysis, it was therefore preferable to ascertain the number of transgene in the range of TAL lines produced. Transgenic plants having a single copy of the transgene are desirable since they show more uniformity and transgene expression is likely to be more stable in subsequent generations (Windels *et al.*, 2003; Peach & Velten, 1991; Tang *et al.*, 2007). The seeds of the self-pollinated T_1 plants were plated onto the medium containing hygromycin. The progeny of T_1 plants (T_2 lines) showing a 3 to 1 ratio was selected for further analysis by counting the number of hygromycin resistant and sensitive seedlings about 2-3 weeks after germination. Three out of 8 T_1 seedlings of *talA* and *talB* transgenic lines showed a 3 to 1 segregation, which were lines A3, A4, A6**,** B5, B6 and B8. These seedlings were transferred to pots containing compost for further analysis. Immunoblotting and transaldolase activity were verified again in T_2 plants to ensure that the over-expressed *E. coli* TAL was inherited and stable in T_2 generation. *Fig. 4* shows strong immuno-reactions between protein extract and antibodies against *E. coli talA* or *talB,* which ensure that T_2 plants inherit normally the expression of *E. coli* TAL from T_1 and also confirm that the expression of *E. coli* TAL is stable in T_2 plants. The total transaldolase activity in T_2 transgenic plants is about 3 to 11 folds higher than that of the wild type. Similarly, the transgenic lines also showed positive correlations between band intensity and total transaldolase activity ($r=0.895$, $p<0.01$ in *talA* plants and $r=0.796$, $p<0.01$ in *talB* plants) (*Fig. 4*), suggesting that both expressed proteins were functional.

Selected T_2 independent lines with single TAL insertion were grown to produce seed, while T_3 homozygous lines were identified

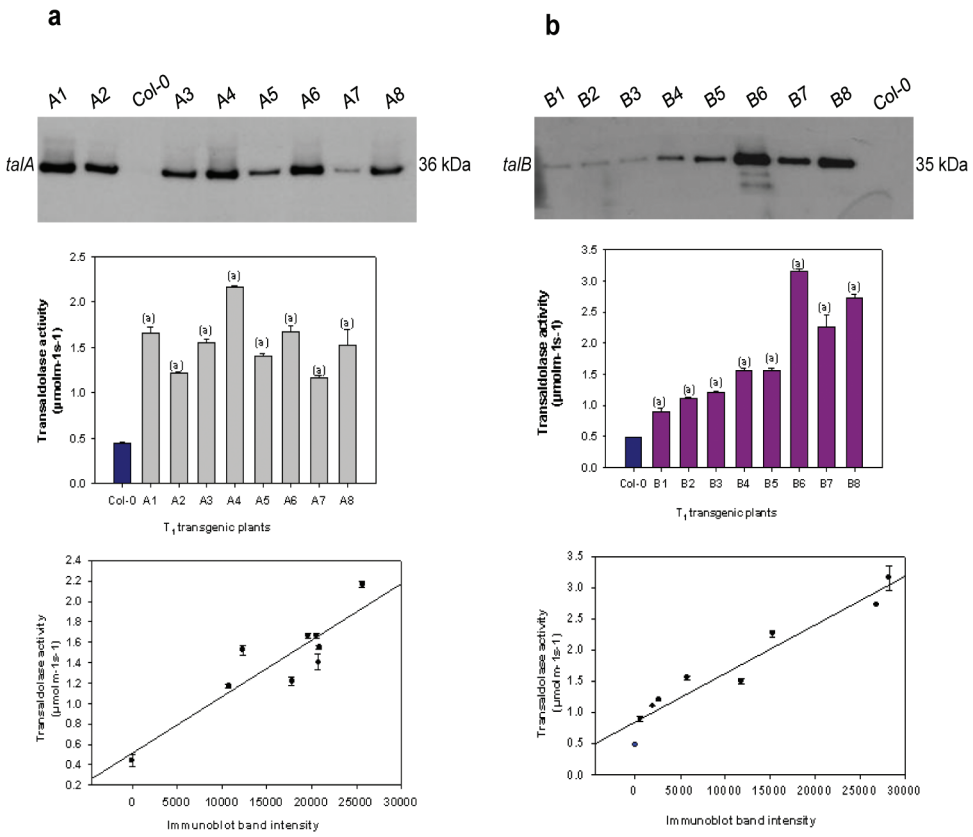

Fig. 3: Immunoblotting, total transaldolase activity and the correlation between total transaldolase activity and immunoblot band intensity of putative T_1 transgenic plants; (a) Putative T_1 transgenic plants over-expressing E. coli talA *in the cytosol; (b) Putative T_1 transgenic plants over-expressing* E. coli talB *in the cytosol. The activity was measured at Week 6 after planting. Values representing the mean and standard errors of the assays were derived from at least three replications [ANOVA and Tukey multiple comparison test ($p<0.05$) and labelled with the letter code (a)]*

after screening on the selective plate (100 % germination). A total 10 of 13 independent homozygous lines were obtained from the batch of the plants transformed with the *E. coli talA* over-expression construct (lines A3-1, A3-2, A3-6, A4-1, A4-3, A4-4, A4-5, A4-6, A6-1, A6-2, A6-3, A6-4 and A6-6). Similarly, 10 independent single insertion and homozygous lines were obtained from the batch of the plants transformed with the *E. coli talB* over-expression construct (lines B5-5, B5-6, B5-7, B6-1, B6-5, B6-6, B8-1, B8-2, B8-6 and B8-7). All of the transgenic lines looked similar to the wild type plants and did not appear to have any distinct visible phenotype. In particular, the height of the plant is similar to that of the wild type, while flowering time and the leaves started senescing at about the same week as the wild type (*Fig. 4c*).

CONCLUSION

Over-expression of *E. coli* TAL in the cytosol of *A. thaliana* was successful. The expression of *E. coli* TAL was stable and functional from

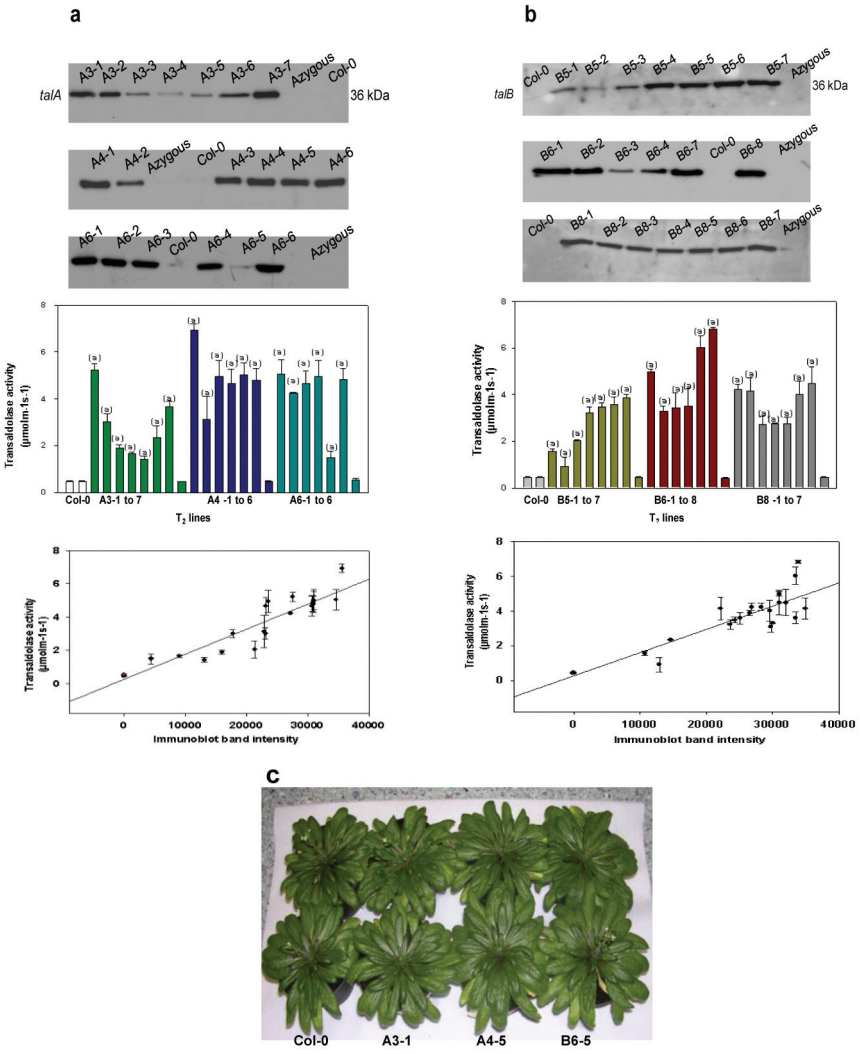

Fig. 4: Immunoblotting, total transaldolase activity, the correlation between total transaldolase activity and immunoblot band intensity and the phenotype of T_2 transgenic lines of E. coli *TAL over-expressed in the cytosol; (a) T_2 lines of* E. coli talA *over-expressed in the cytosol; (b) T_2 lines of* E. coli talB *over-expressed in the cytosol.* A. thaliana *Col-0 (wild type) and azygous lines did not react with the antibody. The activity was measured at Week 6 after planting. Values representing the mean and standard error of assays were derived from at least three replications [ANOVA and Tukey multiple comparison test ($p<0.05$) and labelled with the letter code (a)]; (c) the phenotype of transgenic lines*

T_1 to T_2 generations. Nonetheless, a complete compartmentation in the cytosol of plant did not affect plant phenotype.

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Diversity of *Fusarium* species Isolated from Soil Cultivated with Cucurbits within East Coast, Peninsular Malaysia

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ABSTRACT

Fungi in the genus *Fusarium* are well known as soil-borne pathogen with worldwide distribution. Therefore, this study focused on isolation of *Fusarium* species from soil cultivated with watermelons, muskmelon, pumpkins, and cucumber in the east coast of Peninsular Malaysia by using dilution plate technique, direct plating and debris plating. The highest number of *Fusarium* species isolated was *F. oxysporum* with 687 (26.2%) colonies counted based on colony formation unit (CFU); the colonies of *Fusarium*/g soil = mean of *Fusarium* colonies x dilution factor/weight of dried soil (g). Other *Fusarium* species isolated were *F. semitectum*, *F. solani*, *F. proliferatum*, *F. subglutinans* and *F. chlamydosporum*. Throughout the studies, peptone pentachloronitrobenzene (PPA) medium, potato dextrose agar (PDA) and carnation leaf-piece agar (CLA) were regularly used to identify each *Fusarium* species by morphological means. Based on the Shannon-Weiner Index, *Fusarium* species diversity is much higher in Besut, Terengganu (H'=1.59). *Fusarium* species can be considered as a functionally important biological component of Fusarium fruit rot disease study in cucurbits.

Keywords: ***Fusarium, rot disease, cucurbits, Soil Microbiology***

INTRODUCTION

Fusarium species cause a huge range of diseases on an extraordinary range of host plants. In the Family of Cucurbitaceae (e.g. watermelons, muskmelon, pumpkins, and cucumber), it can cause diseases known as vascular wilt, root rot and fruit rot (Vakalounakis & Chalkias, 2004). *Fusarium* is a genus of deuteromycetous fungi which are abundance in soils, whole parts of a plant, plant debris and other organic substrates (Summerell *et al*., 2003). It is found to be well and important genera living in the soils as free-living saprophytes, pathogens, and endophytes and known to produce a range of toxic compounds that can adversely affect livestock and humans (Summerell *et al*., 2001). Soil fertility is a main factor for the occurrence and virulence of *Fusarium* species. Meanwhile, Nik Mohd Izham *et al*. (2005) reported that *Fusarium* species are well distributed in soils planted with crops such as paddy, rubber, oil palm and vegetables compared to the unused

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soils. A previous study by Lim (1971) was the first to report on the diversity of *Fusarium* species in Malaysian soils which had isolated 8 species with *F. solani* the widest spread species, and this was followed by *F. oxysporum*. It has been reported that various *F. oxysporum* pathotypes can survive successfully either in the soil or above ground by means of thick-walled chlamydospores that are either free or embedded in infected plant debris (Suarez-Estrella *et al*., 2004). Thus, this study was undertaken to determine: (i) *Fusarium* species isolated from the soils by using morphological identification, and (ii) its diversity in east coast, Peninsular Malaysia, especially the areas that are highly cultivated with cucurbits such as in Bachok, Pasir Mas, Tok Bali (Kelantan district), as well as Besut and Setiu (Terengganu district).

MATERIALS AND METHODS

Soil Samples

In this study, the soil samples were collected from areas cultivated with cucurbits (watermelon, muskmelon, pumpkin and cucumber) around the east coast of Peninsular Malaysia (Kelantan and Terengganu). Field sampling was done between January 2009 and April 2009. The soil samples were air-dried at room temperature (27±1°C) for 5 days and ground. The ground soil was then sieved with 0.5 mm sieve to separate larger particles such as debris. Both the soil and debris were kept in paper envelopes.

Isolation of Fusarium spp.

The isolation was based on three methods; namely soil dilution plate, soil direct plating and debris plating, as described by Leslie and Summerell (2006).

Soil Dilution Plate Technique

One ml of the soil suspension that had been diluted from 10^{-2} to 10^{-4} was spread on the surface of peptone pentachloronitrobenzene (PPA) medium with 7 replicates for each dilution factor. The dilution plates were observed daily for 7 days for colony counting. *Fusarium* spp. colonies were counted based on the formula below for colony formation unit (CFU) (Nash & Snyder, 1962):

$$\text{Colonies of } \textit{Fusarium}\text{/g soil} = \frac{\text{Means of } \textit{Fusarium} \text{ colonies x dilution factor}}{\text{Weight of dried soil (g)}}$$

Soil Direct Plating

10 mg of the sieved soil were spread evenly on the surface of PPA medium and incubated. This was followed by observation within 7 days for colonies formation.

Debris Isolation Technique

After soil sieving, the collected debris was suspended in the running water for 24 hours with sieve as a separator to remove the soil particles that might be attached to debris. Then, the debris was air-dried on sterile paper and placed on the surface of PPA medium. Both direct plating and debris plating techniques were used for the qualitative data.

All *Fusarium* spp. that were isolated from those three techniques were then identified on PDA and CLA. The identification was based on the morphological characteristics from Leslie and Summerell (2006).

Measurement of Species Diversity

Fusarium species diversity within the east coast of Peninsular Malaysia was calculated by using the Shannon-Weiner Index (Spellerberg, 2008):

$$H' = -\sum_{i=1}^{s} p_i \ln p_i$$

where: $\sum$ refers to "the sum of"
there are *s* species in the community
p_i = is the relative abundance (proportion) of the i species in the community
ln = natural log

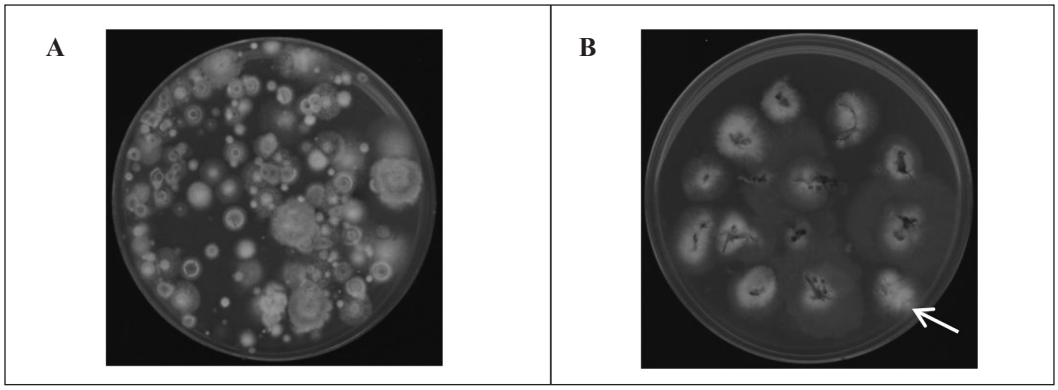

Fig. 1: (A) Colonies of Fusarium *species from soil dilution plate technique on PPA medium; (B)* Fusarium *species from debris on PPA medium (arrow)*

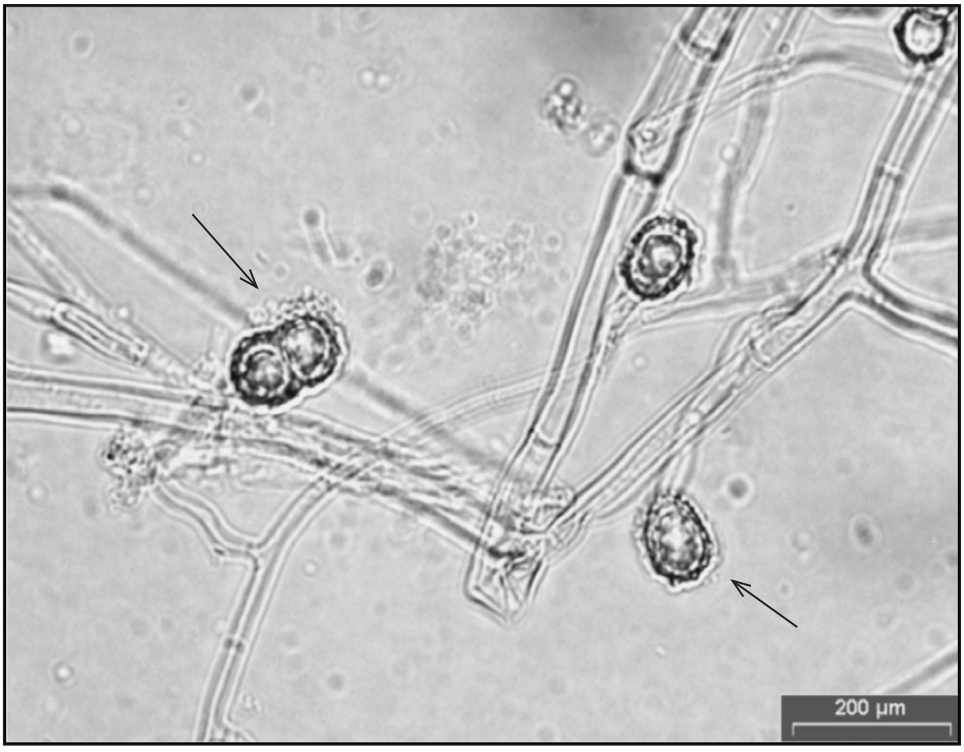

Fig. 2: The thick-walled chlamydospores of F. oxysporum *(arrow) that is either free or embedded in infected plant debris*

TABLE 1
Number of *Fusarium* species colonies isolated per gram soils (CFU/g soil) by using soil dilution plate (10^{-2}) technique and species diversity (Shannon-Weiner Index)

Soil Locality	*Fusarium* species colonies						$p_i \ln p_i$
	[1]*F. chl*	[2]*F. oxy*	[3]*F. pro*	[4]*F. sem*	[5]*F. sol*	[6]*F. sub*	
Bachok, Kelantan	0	55	75	142	71	48	1.52
Pasir Mas, Kelantan	0	146	185	122	74	115	1.57
Tok Bali, Kelantan	24	101	34	114	65	5	1.50
Besut, Terengganu	18	190	74	132	191	53	1.59
Setiu, Terengganu	1	195	79	121	159	35	1.49
Total	43	687	447	631	560	256	

[1]*F. chlamydosporum*, [2]*F. oxysporum*, [3]*F. proliferatum*, [4]*F. semitectum*, [5]*F. solani*, [6]*F. subglutinans*

RESULTS AND DISCUSSION

Only the number of colonies from the dilution factor 10^{-2} was chosen as an optimum concentration of soil dilution for CFU counting on PPA medium. After five days, more *Fusarium* spp. colonies appeared in the PPA medium from soil dilution plate technique (*Fig. 1a*) and also debris (*Fig. 1b*). A total of 2,624 *Fusarium* colonies with 26.2% *F. oxsporum* was isolated, and this was followed by *F. semitectum* (24.0%), *F. solani* (21.3%), *F. proliferatum* (17.0%), *F. subglutinans* (9.8%) and *F. chlamydosporum* (1.7%).

The highest number of *Fusarium* species colonies isolated from the soil cultivated with cucurbits was *F. oxysporum,* with a total of 687 colonies (Table 1). Based on the Shannon-Weiner Index, even though all five locations in the east coast of Peninsular Malaysia had the same species richness, the *Fusarium* species diversity was found to be much higher in Besut, Terengganu (H'=1.59), followed by Pasir Mas, Kelantan (H'= 1.57), Bachok, Kelantan (H'= 1.52), Tok Bali, Kelantan (H'= 1.50) and Setiu, Terengganu (H'= 1.49) (Table 1).

The survival of *F. oxysporum* in soil and debris has been proven by Vakalounakis and Chalkias (2004), whereby it was found that *F. oxysporum* f. sp. *radicis-cucumerinum* could survive as a successful soil-inhabiting fungus for more than 13 months and cause root and stem rot of cucumber. *F. oxysporum, F. semitectum, F. solani and F. chlamydosporum* produced chlamydospores (*Fig. 2*) that may survive successfully in plant debris and could act as inoculums source when the environment is suitable for dispersion from one growing season to the next. Cucurbits fruits attached to the soil were easily infected with Fusarium fruit rot (FFR) disease; a recent study showed that 54% of *F. solani* and 46% of *F. oxysporum* were isolated from the east coast of Peninsular Malaysia, i.e. from the samples with FFR disease (Siti Nordahliawate *et al*., 2009).

Meanwhile, some research has focused on the survival of various *F. oxysporum* pathotypes in soils. Among other Roncero *et al.* (2003) used *Fusarium* as a model system to understand the process of root infection and disease development in soil-borne plant pathogens. Their ubiquitous distribution in soil may contribute as saprophytic decomposition in the process of nutrients cycling, while some species such as *F. oxysporum* have been found to beneficial in soil denitrification (Takaya *et al.,* 2002; Steven *et al*., 2008). These studies would become a platform for researchers and students to understand the importance of *Fusarium* species as a soil-borne microorganism that might contribute to the economically important cucurbit disease in the east coast of Peninsular Malaysia. In addition, soil treatments, crop rotation and other measure controls can be considered to control the diseases caused by *Fusarium* species, specifically at the highest inoculums area.

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Journal of Tropical Agricultural Science

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