Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

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Pertanika Journal of Tropical Agricultural Science
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About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science is an official journal of Universiti Putra Malaysia. It is an open-access online scientific journal. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognised internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

Pertanika Journal of Tropical Agricultural Science is a **quarterly** (*February*, *May*, *August*, and *November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open for submission by authors from all over the world.

The journal is available world-wide.

Aims and scope

Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

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Pertanika was founded in 1978. A decision was made in 1992 to streamline *Pertanika* into 3 journals as Pertanika Journal of Tropical Agricultural Science, Pertanika Journal of Science & Technology, and Pertanika Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

Currently, as an interdisciplinary journal of agriculture, the revamped journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

Vision

To publish journals of international repute.

Mission

Our goal is to bring the highest quality research to the widest possible audience.

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 90 days. The elapsed time from submission to publication for the articles averages 180 days. We are working towards decreasing the processing time with the help of our editors and the reviewers.

Abstracting and indexing of Pertanika

Pertanika is now over 42 years old; this accumulated knowledge has resulted in Pertanika Journal of Tropical Agricultural Science being abstracted and indexed in SCOPUS (Elsevier), Clarivate Web of Science (ESCI), EBSCO, DOAJ, Agricola, ASEAN CITATION INDEX, ISC, Microsoft Academic, Google Scholar, National Agricultural Science (NAL), and MyCite.

Citing journal articles

The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci*.

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Pertanika policy prohibits an author from submitting the same manuscript for concurrent consideration by two or more publications. It prohibits as well publication of any manuscript that has already been published either in whole or substantial part elsewhere. It also does not permit publication of manuscript that has been published in full in proceedings.

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The *Pertanika* journals and Universiti Putra Malaysia take seriously the responsibility of all its journal publications to reflect the highest publication ethics. Thus, all journals and journal editors are expected to abide by the journal's codes of ethics. Refer to *Pertanika*'s **Code of Ethics** for full details, or visit the journal's web link at http://www.pertanika.upm.edu.my/code_of_ethics.php

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Lag time

A decision on acceptance or rejection of a manuscript is expected within 90 days (average). The elapsed time from submission to publication for the articles averages 180 days.

Authorship

Authors are not permitted to add or remove any names from the authorship provided at the time of initial submission without the consent of the journal's Chief Executive Editor.

Manuscript preparation

Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words ***I****ntroduction*, ***M****aterials and Methods*, ***R****esults*, ***A****nd* ***D****iscussion*. IMRAD is simply a more 'defined' version of the "IBC" (*Introduction*, *Body*, *Conclusion*) format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title*, *Authors*, *Keywords*, *Abstract*, *Conclusions*, *References*, and *Acknowledgement*. Additionally, some papers include *Appendices*.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the journal's **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_ Authors.pdf).

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Authors who complete any submission are notified with an acknowledgement containing a manuscript ID on receipt of a manuscript, and upon the editorial decision regarding publication.

Pertanika follows a double-**blind peer review** process. Manuscripts deemed suitable for publication are sent to reviewers. Authors are encouraged to suggest names of at least 3 potential reviewers at the time of submission of their manuscripts to *Pertanika*. The editors are not, however, bound by these suggestions.

Notification of the editorial decision is usually provided within 90 days from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

As articles are double-blind reviewed, material that may identify authorship of the paper should be placed only on page 2 as described in the first-4-page format in *Pertanika*'s **Instruction to Authors** (http://www.pertanika.upm.edu.my/Resources/regular_issues/Regular_Issues_Instructions_to_Authors.pdf).

The journal's peer review

In the peer review process, 2 or 3 referees independently evaluate the scientific quality of the submitted manuscripts. At least 2 referee reports are required to help make a decision.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are 7 steps to the editorial review process:

1. The journal's Chief Executive Editor and the Editor-in-Chief examine the paper to determine whether it is relevance to journal needs in terms of novelty, impact, design, procedure, language as well as presentation and allow it to proceed to the reviewing process. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The Chief Executive Editor sends the article-identifying information having been removed, to 2 or 3 reviewers. They are specialists in the subject matter of the article. The Chief Executive Editor requests that they complete the review within 3 weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the research field.

3. The Editor-in-Chief examines the review reports and decides whether to accept or reject the manuscript, invite the authors to revise and resubmit the manuscript, or seek additional review reports. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the authors) are forwarded to the authors. If a revision is indicated, the editor provides guidelines to the authors for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the Chief Executive Editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The authors may also submit a rebuttal if there is a need especially when the authors disagree with certain comments provided by reviewers.

5. The Chief Executive Editor sends the revised manuscript out for re-review. Typically, at least 1 of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the Editor-in-Chief examines their comments and decides whether the manuscript is ready to be published, needs another round of revisions, or should be rejected. If the decision is to accept, the Chief Executive Editor is notified.

7. The Chief Executive Editor reserves the final right to accept or reject any material for publication, if the processing of a particular manuscript is deemed not to be in compliance with the S.O.P. of *Pertanika.* An acceptance notification is sent to all the authors.

   The editorial office ensures that the manuscript adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the editorial office. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the manuscript appears in the pages of the journal and is posted on-line.

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ABSTRACTING AND INDEXING OF PERTANIKA JOURNALS

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Contents

Foreword

Welcome to the Second Issue of 2021 for the Pertanika Journal of Tropical Agricultural Science (PJTAS)!

PJTAS is an open-access journal for studies in Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university for the benefit of the world-wide science community.

This issue contains 13 articles; 12 are regular articles and a review article. Articles submitted in this issue cover the scope of aquaculture; biotechnology; botany; crop and pasture production; food and nutrition development; forestry sciences; food and nutrition development; forestry sciences; genetics and molecular biology; microbiology; soil and water sciences; microbiology; plant physiology; plant physiology; and zoology. The authors of these articles come from different countries namely Indoneisa, Malaysia, and Thailand.

A regular article entitled "Protocols for the Extraction of High-quality RNA from Pineapple Tiller, Flower, Inflorescence, and Fruits" reported the protocols for the extraction of high-quality RNA from two types of pineapple tissues, which are thickly lignified hard tissue and watery soft tissue via modified Kim and Hamada (2005) method. High-quality RNA, which is suitable for subsequent molecular analysis, was successfully extracted through this modified method. Full information of this study is presented on page 293.

A selected article entitled "The Effects of Sago (*Metroxylon sagu*) Bark and Frond Waste as Substrates on the Growth and Yield of Grey Oyster Mushrooms (*Pleurotus sajor-caju*)" examined the effects of sago bark (SB) and sago frond (SF) waste on the growth and yield of grey oyster mushrooms (*Pleurotus sajor-caju*). The results showed that SB and SF can be utilised and further developed for the cultivation of oyster mushroom by replacing the usage of sawdust (SD). The detailed information of this article is available on page 307.

Budi Setiadi Daryono and his teammates from Gadjah Mada University investigated the screen house effect on *Begomovirus* diversity severity and coat protein diversity in chili. They concluded that optical manipulation using a UV screen or a screen house was effective in reducing either *Begomovirus* infection or whitefly population. These findings may serve as the alternatives to the development of resistant cultivars for controlling *Begomovirus* infections. The further details of this study are found on page 449.

We anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

All the papers published in this edition underwent Pertanika's stringent peer-review process involving a minimum of two reviewers comprising internal as well as external referees. This was to ensure that the quality of the papers justified the high ranking of the journal, which is renowned as a heavily-cited journal not only by authors and researchers in Malaysia but by those in other countries around the world as well.

We would also like to express our gratitude to all the contributors, namely the authors, reviewers, Editor-in-Chief and Editorial Board Members of PJTAS, who have made this issue possible.

PJTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

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Review Article

Plant Growth-promoting Microorganisms Isolated from Plants as Potential Antimicrobial Producers: A Review

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ABSTRACT

The agricultural industry worldwide faces challenges in the struggle against plant diseases. In efforts to increase agricultural intensities, the dependency on agrochemicals for crop protection has become significantly high. Moreover, the increasing use of agrochemical-based products has resulted in multidrug-resistant pathogens and environmental pollution. This paper reviews the biocontrol capacity of plant growth-promoting microorganisms (PGPMs) originating from plants towards plant pathogens. The current trend in discovering new compounds has shown antimicrobial activity gaining immense interest due to its vast potential. On a related note, PGPMs are an aspect of that research interest that can be further explored as antimicrobial producers. In this work, the types of biocontrol mechanisms pertaining to PGPMs as well as their roles in biocontrol activity were covered. A biocontrol approach exploits disease-suppressive microorganisms to improve plant health by controlling related pathogens. The understanding of these microorganisms and mechanisms of pathogen antagonismare primary factors in ensuring improvement for future applications. Inevitably, there is indeed room for rigorous expansion with respect to PGPMs in the future of agriculture.

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INTRODUCTION

Infections caused by microorganisms such as bacteria, fungi, and parasites are known to be significant problems for humans, animals, and plants. The discovery of penicillin in 1928 revealed that microorganisms may contribute to known existing issues, due to the abundant bioactive substances produced within their cells (L.-Q. Xu et al., 2017). Some pathogens have been shown to develop antimicrobial-resistant properties, leading to increased morbidity, mortality, and healthcare costs (Pratiwi et al., 2017).

In food and agriculture, the use of metals, heavy metals, and biocides have both direct and indirect impacts on the growth of antimicrobial resistance in bacteria that can invade the food chain (Cheng et al., 2019). As described by Horrigan et al. (2002), traditional agriculture, includes high-yielding plants, mechanised tillage, inorganic fertilisers, and chemical pesticides, which have in turn lead to problems such as biodiversity loss (Hole et al., 2005) and impacts on soil biota and related health problems (Aktar et al., 2009). In order to boost crop yields in a sustainable and environmentally responsible manner, most of these current agricultural methods that require the use of chemical fertilisers, herbicides, fungicides, and insecticides need to be re-examined (Glick, 2012).

This scenario leads to increased research focused on the development of new antibiotics and bioactive compounds in the fight against multidrug-resistant microbes (Bérdy, 2005) and biocontrol agents against plant diseases (Köhl et al., 2019). The use of PGPMs as a biological control agent in the form of biopesticides has been considered one of the best practices for the sustainability of agroecosystems as they provide solutions to issues such as resistance to pests, conventional chemical pesticides, and public concern regarding the side effects of pesticides on the environment and eventually on human health (Mishra et al., 2015). A study by Moin et al. (2020) showed a suppression of root rotting fungi by 55.5% in 2017, and 63.6% in 2018 in sunflowers as compared to other treatments, including carbendazim, a commercial fungicide. Therefore, the use of PGPMs in the management of plant diseases has emerged as a new future alternative.

Moreover, there is an abundance of possible microorganism sources to attain biocontrol for plant growth. PGPMs, such as rhizobia, mycorrhizae, and plant growth-promoting bacteria, have been documented for decades to boost plant growth under stressed and non-stressed conditions (Naamala & Smith, 2020). According to Bérdy (2005), these would include endophytes. Prashar et al. (2013) and Rekha et al. (2010) also included rhizobacteria for this purpose. In brief, PGPMs indeed possess the capacity to serve as biocontrol agents against plant pathogens, and in addition they are effective in preserving the soil quality and increasing crop yield in a sustainable approach.

Plant Growth-Promoting Microorganisms

Plants are perceived as meta-living beings

with a particular microbiome and have advantageous interactions and associations with related microorganisms (Mendes et al., 2013). A greater understanding of the interactions of a plant with its microbiome has improved knowledge of its ability to affect its microbiome and vice versa (Jones et al., 2019). In natural settings, plants are associated with a microbial population in healthy tissues (Darma et al., 2016). PGPMs are in fact central to microbial communities. PGPMs have been associated with several types of plants and are commonly found in many environments. In general, PGPMs can be divided into two main groups: plant growth-promoting (rhizo)bacteria (PGPRs or PGPBs) and plant growth-promoting fungi (PGPFs).

A typical example of plant growth-promoting bacterium (PGPBs) is the beneficial free-living soil bacteria (Agrillo et al., 2019). PGPBs are known to potentially occupy various plant compartments, including the rhizosphere, the endosphere (inner plant tissues), and the phyllosphere (an aerial portion of plant leaves) (Zhang et al., 2019). Rhizobacteria, among a variety of other microorganisms, often populate the rhizosphere, a small area of soil directly affected by the root system. This may be due to the abundance of nutrients and energy sources produced by different plant exudates, such as amino acids and sugars, compared to the bulk soil region (Gray & Smith, 2005).

According to Cecagno et al. (2015), the genus *Azospirillum*, a known PGPR, is comprised of free-living, nitrogen-fixing bacteria, which can colonise the root surface or the intercellular spaces of the host plant roots by adhesion. The advantages of *Azospirillum* inoculation for plants were mainly due to its ability to fix atmospheric nitrogen (Fukami et al., 2018) and the ability to secrete phytohormones such as auxins, gibberellins, cytokinins, and nitric oxide, which promote plant growth (Fibach-Paldi et al., 2011).

The definition of PGPF is equivalent to that of PGPR, according to Murali and Amruthesh (2015), except that the species are fungi (including true fungi as well as oomycetes) rather than bacteria. PGPFs have the ability to provide plants with many benefits in terms of growth and defence against pests and pathogens (Hossain et al., 2014). Larran et al. (2016) described a *Fusarium* sp. that was isolated from wheat that showed a significant reduction in spore germination of *Drechsleratritici-repentis*, which causes tan spot of wheat, to be suppressed by 52% compared to the control. Another study by Fiume and Fiume (2008) identified the inhibition of *Pyrenochaeta lycopersici*, which causes corky root in tomato, by *Trichoderma viride* 18/17 SS, which increased up to 81.2% in dual culture. In addition, the study also proved that all tomato plants treated with *T. viride* showed significant differences from untreated tomato plants with regards to corky root symptoms during growth in a greenhouse in which the McKinney index rose from 3.3 to 23.3%.

In addition, numerous endophytic microorganisms have also been classified

as PGPMs and are defined as part of the plant microbiota (Zhang et al., 2019). These microorganisms are classified as non-pathogenic bacteria or fungi that live in healthy living tissues of plants but do not inflict any damage to the plants (Bacon & White, 2000). As described by W. Xu et al. (2019), these endophytes are isolated from various plant species, and some of them may have the potential to be utilised as biocontrol agents against plant diseases such as white fruit disease which usually threatens mulberry fruit productivity. Moreover, endophytes have also been shown to stimulate the growth of mulberry seedlings. The results showed that the highest promotion potential was caused by *Bacillus* sp. CW16-5, which increased shoot length and root fresh weight by 83.37% and 217.70%, respectively (W. Xu et al., 2019).

Previous reports have shown that endophytes can aid germinating seeds and improve plant growth through several approaches such as nitrogen fixation, phosphate solubilisation, siderophore production, and bioactive enzyme release (Agrillo et al., 2019; Liotti et al., 2018; Wu et al., 2020; W. Xu et al., 2019). Furthermore, W. Xu et al. (2019) mentioned another promising source of natural biological control antagonists (BCAs). Endophytic bacteria are considered natural BCAs due to their production of possible bioactive substances. In addition, compared to soil-derived fungi or other settings, endophytic fungi have a greater affinity mostly with the host and can more easily infect and flourish in plants, thereby offering better resistance and desirable effects on plants (Backman & Sikora, 2008).

Biocontrol Mechanism of PGPMs

Biocontrol of plant diseases can be described as suppressing plant-pathogen populations using living organisms (Heimpel & Mills, 2017). Biocontrol agents use different mechanisms to shield plants from pathogens (Köhl et al., 2019), which able to reduce the use of agrochemicals in agricultural production (Naamala & Smith, 2020). As described by Sehrawat and Sindhu (2019), many rhizobacteria produce an antagonistic effect by using diverse mechanisms of biocontrol including creating a competitive environment for nutrient uptake against the phytopathogenic microorganisms, the root colonisation ability and producing a secondary metabolite as protective agents. In addition, the rhizobacteria also help in regulating the production of virulence factor using quorum sensing and inducing a physical defence mechanism of the host such as induced systemic resistance (ISR) and systemic acquired resistance (SAR) as a mechanism of biocontrol (Sehrawat & Sindhu, 2019).

Beneduzi et al. (2012) identified PGPBs that indirectly suppress the activity of phytopathogens based on competition for living capacity (space and nutrients) or the development of antibacterial metabolites (Beneduzi et al., 2012). This metabolite is responsible for the antagonistic action of certain species against phytopathogens such as *Alternaria solani* (Attia et al., 2020), *Aspergillus flavus* (Chen et al., 2019), and

Fusarium solani (Bahroun et al., 2018). Several studies have documented the use of lytic enzymes, such as chitinases and proteases, in addition to antimicrobial peptides or proteins, polyketides, phenolic compounds, and biosurfactants (Abdalla et al., 2020; Agrillo et al., 2019; Chen et al., 2019; Wu et al., 2020; W. Xu et al., 2019; Zloch et al., 2016), in the development of antimicrobial metabolites.

PGPBs may also implement other biocontrol mechanisms to directly enhance plant development, such as biofilm production (Naik et al., 2015), nutrient uptake, nitrogen fixation (Carvalho et al., 2014), mineral phosphate solubilisation (Wang et al., 2017), phytohormones, and siderophore release (Gamalero & Glick, 2015). A study by Chen et al. (2019), also found that the endophyte *Bacillus velezensis* LDO2 produces specific metabolites such as fengycin, surfactin, bacilysin, bacillaene, and macrolactin, which constitute the basis for pathogen inhibition. Other strategies involve altering the cell membrane permeability of the pathogen, triggering cell lysis, and producing siderophores that can minimise pathogen growth by reducing iron accessibility (Zloch et al., 2016).

There is also a non-pathogenic soil *Bacillus* spp. with the ability to form endospores. *Bacillus* spp. have been found to colonise root surfaces, whereby they enhance plant growth by triggering fungal mycelia lysis (Turner & Backman, 1991). This strain is advantageous as it can withstand intense pH, temperature, and osmotic conditions (Ashwini & Srividya, 2013). *Bacillus* spp. are also considered safe biological agents, leading to their higher potential of use, due to various actions, including antibiosis, siderophore production, cell wall degrading enzymes, and lipopeptide producers (Islam et al., 2012).

Generally, PGPMs can promote plant growth via both direct and indirect mechanisms. Direct mechanisms are characterised by employing the bacterial traits that directly promote plant growth, while indirect mechanisms refer to bacterial traits that prevent one or more plant pathogenic organisms from functioning (Olanrewaju et al., 2017). By using either one or more of these mechanisms, PGPMs can influence plant growth and development. In the remainder of this review, the mechanisms of action of biocontrol agents and identify some promising examples of these PGPMs in controlling plant disease would be dicussed. Figure 1 provides application of PGPMs in biocontrol activity and their mode of action.

Antimicrobial Secondary Metabolites as Antibiosis Agents. Compant et al. (2005) described an essential mechanism in biological control as the production of antimicrobial secondary metabolites. Pathogens tend to interact with microbial biological control agents via antibiosis or hyperparasitism to assure crop yields (Köhl et al., 2019). This can be seen in the number of beneficial rhizobacteria capable of secreting antibiotics and other compounds that are antagonistic to plant pathogens. *Agrobacterium*, *Bacillus*, *Burkholderia*,

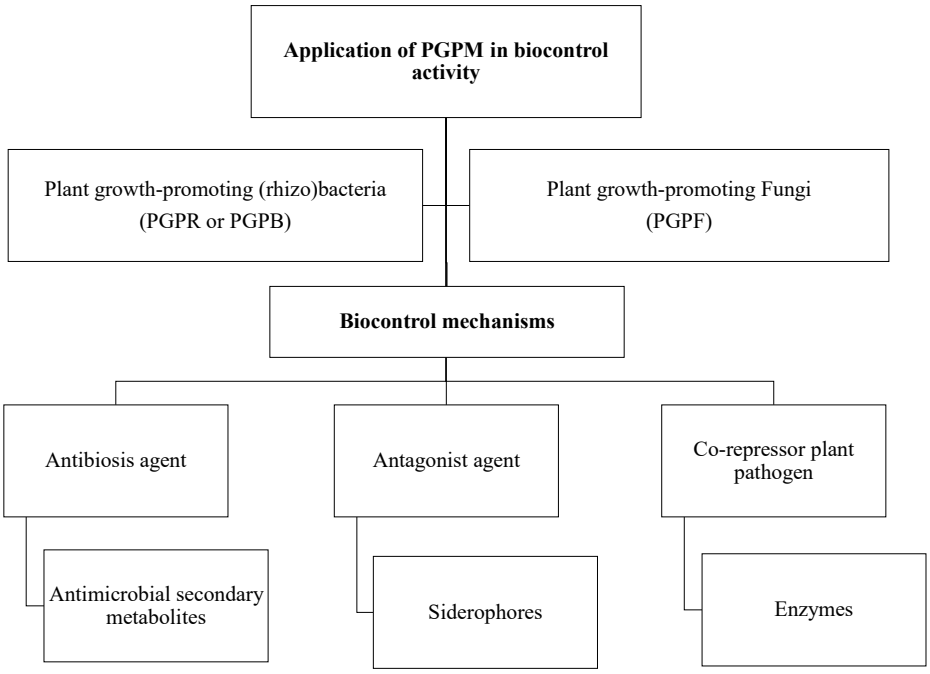

Figure 1. Application of PGPMs in biocontrol activity and their mode of action

Pseudomonas, and *Streptomyces* are effective antagonists against soil-borne pathogens (Barea et al., 2005; Montealegre et al., 2003; Prapagdee et al., 2008). These have been shown to be broad-spectrum antimicrobial producers against *Aeromonas hydrophila*, *Escherichia coli*, and *Staphylococcus aureus* (Vachee et al., 1997), *Bacillus subtilis*, *Candida albicans*, and *Proteus vulgaris* (Trujillo et al., 2007). This remarkable feature was further highlighted by Rekha et al. (2010) such that *Pseudomonas* spp. are also pertinent as root dips for biological control of soil-borne plant pathogens, seed inoculants, and antibacterial agents.

Essentially, there is a significant correlation between antimicrobial development and disease inhibition. For instance, *Pseudomonas* spp. can yield antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG), which regulates and reduces *Gaeumanomyces graminis* var. *ritici* infection in wheat as described by de Souza et al. (2003). Moreover, in the field trials by Weller (2007), a 60% suppression of pathogen infection was documented from the bacterization of wheat seed with antibiotic phenazine-1-carboxylic acid (PCA) activity by *Pseudomonas fluorescens* strains 2-79. Most *Bacillus* spp., as well as many other pathogenic fungi such as *Alternaria solani*, *Aspergillus flavus*, *Botryosphaeria ribis*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Helminthosporium maydis*, and *Phomopsis gossypii* synthesise antibiotics, namely

polymyxin, circulin, and colistin, which are active against gram-positive and gram-negative bacteria (Maksimov et al., 2011). Meanwhile, Cawoy et al. (2014) reported that the metabolites secreted by the *Bacillus* genus resulted in an antagonism effect responsible for the disruption of the fungal membrane, resulting in mycelium, conidia, or zoospores for oomycete pathogens. These metabolites are extensively used as biocontrol agents for their antibacterial and antifungal properties, particularly surfactin, iturin, plipastatin, and fengycin (Harwood et al., 2018).

In addition, endophytes are also capable of producing a wide variety of biologically active secondary metabolites. For example, Vaz et al. (2009) reported endophytic fungi isolated from a tropical orchid extract have shown to demonstrate the strongest antimicrobial activities against the pathogenic yeasts and may be considered to produce bioactive natural products. J. C. Lee et al. (1995) further added that hydroxypestalopyrone, pestaloside, pestalopyrone, and pyrone are some examples of antifungal compounds produced by isolated endophytic *Pestalotiopsis microspora* from *Torreya taxifolia*. From the Sepik River region in Papua New Guinea, isolated endophytic *Pestalotiopsis jester* have been reported to produce antifungal jesterone and hydroxy-jesterone that counter multiple phytopathogenic fungi (Li & Strobel, 2001). Other work by W. Xu et al. (2019) also mentioned broad-spectrum antagonism on phytopathogens demonstrated by four strains of *Pantoea* spp. (CA15-30, CA15-43, CA15-44, and XA15-46), one strain of *Pseudomonas* spp. (XA15-33), and 26 isolates of *Bacillus* spp. This activity can easily be associated with antibiotic biosynthesis.

Siderophore Production as Antagonist Agents. A limiting factor in iron availability for microbial growth is due to the low solubility of Fe^{3+} ions, yet it is an essential consideration for disease suppression (Köhl et al., 2019). Microorganisms capable of producing high levels of siderophores with high iron affinity may be selected for biological control against phytopathogens. By limiting the level of iron that is usable for a pathogen, siderophore-producing microbes can prevent or minimise pathogen proliferation (Shen et al., 2013), causing them to lose their ability to function as pathogens (Olanrewaju et al., 2017).

In terms of site competition, siderophores are low molecular weight ferric ion specific chelating agents that can be differentiated into three main categories, hydroxamates, catecholates, and carboxylates. As described by Pahari et al. (2017), hydroxymate type of siderophore is mostly produced by the bacteria and fungi. The catecholate siderophore, produced by the bacteria and carboxylate siderophore produced by bacteria lik*e Rhizobium* sp., *Staphylococcus* sp. and fungi like *Mucorales* sp. According to Battu and Reddy (2009), several strains from the *Pseudomonas fluorescens putida* group can release siderophores that are beneficial for plant growth and biocontrol. This may be due to fungal inhibition

within the rhizospheres of several crops. Furthermore, several fungi, such as *Trichoderma asperellum*, also produced iron-binding siderophores to control Fusarium wilt (Segarra et al., 2010). Ahmad et al. (2008) stipulated another noteworthy advantage, aside from acquiring iron, is that siderophore-producing endophytic bacteria can also inhibit phytopathogenic growth by depleting iron. In another study, Calvente et al. (2001) proved the growth inhibition of phytopathogenic moulds by bacterial siderophores containing spent medium and associated the antifungal activity with siderophore concentration.

Enzymes as Co-repressor of Plant Pathogens. In some cases, excretion of cell wall degrading enzymes (CWDEs) supports the biocontrol of secondary metabolites. Cell wall degradation can typically be associated with a range of chitinases, β-1,3-glucanases, and proteases. The biocontrol strains of PGPRs that secrete CWDEs have a strong inhibitory effect on the hyphal growth of fungal pathogens. Enzymes, namely, chitinase and β-1,3-glucanase, degrade chitin, which is the major component of the fungal cell wall (Labuschagne et al., 2010). Nevertheless, for hyperparasites such as oomycota, pathogen cell walls are penetrated by cellulases (Köhlet al., 2019). Xylanases, cellulases, and chitinases are some of the enzymes secreted during colonisation and infection processes. As these enzymes or their degradation products maybe directly recognised by the host, they may induce a defensive response (Druzhinina et al., 2011). *Curtobacterium* sp. XA15-35, demonstrated antifungal activity, which counters *Sclerotinia sclerotium* (W. Xu et al., 2019). This attribute of XA15-35 is associated with significant phosphate solubilisation besides the production of several hydrolytic enzymes (chitinase and protease).

A previous study by Radjacommare et al. (2004) also documented mycelial growth inhibition of *Rhizoctonia solani* from induced resistance activity. *Pseudomonas fluorescens* apparently demonstrated an induced systemic resistance (ISR) to hinder the sheath blight pathogen by the latter, which increased the production of chitinase genes in rice. This example reflects various endophyte-derived compounds that are capable of inducing plant defence responses. However, due to the high complexity of hyperparasitism, the production of a single enzyme may not be a good competitor for biocontrol purposes. In addition, according to Karlsson et al. (2017), understanding the role of enzymes in biocontrol requires the perception of the entire cascade of events, including the signal regulation of its various secondary metabolites. The beneficial microorganisms were found to be able to incorporate ISR to enhance the protective ability of the whole plant to multiple infections (Conrath et al., 2015). For instance, the degree of stunting, leaf malformation, and wilting induced by *Xylella fastidiosa* in *Catharanthus roseus* (Lacava et al., 2007) can be abated by *Curtobacterium flaccumfaciens*.

Table 1

Biocontrol of plant pathogens by plant growth-promoting microorganism and its biocontrol mechanisms against plant pathogen

Biocontrol agent	Target pathogen	Plant/Disease	Mechanism of action	Reference
Bacillus subtilis SBMP4, *Lysinibacillus fusiformis* NBRC15717, *Achromobacter xylosoxidans* NBRC15126	Alternaria solani	Early blight disease (Tomato)	Antibiotics, secondary metabolites, and enzyme	Attia et al. (2020)
Pseudomonas sp. EFP-121	*Macrophomina phaseolina* *Fusarium* spp.	Root rot (Sunflower)	Siderophores, ammonia, HCN, and induced systemic resistance	Moin et al. (2020)
Pseudomonas protegens N	Aspergillus niger	Bunch rot and black mould (Tomato)	Antimicrobial peptide	Agrillo et al. (2019)
Bacillus velezensis LDO2	*Alternaria tenuissima*, *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Rhizoctonia solani*, *Rhizopus* sp.	Leaf blight, damping-off, crown rot, root rot, and pod rot (Peanut)	Siderophores and secondary metabolites	Chen et al. (2019)
Bacillus velezensis OEE1	*Verticillium dahliae*	Verticillium wilt (Olive tree)	Secondary metabolites	Azabou et al. (2020)
Rahnella aquatilis B16C	*Fusarium solani*	Root rot (Faba bean)	Secondary metabolite, siderophores, PRN, and HCN	Bahroun et al. (2018)
Burkholderia stabilis PG159	*Cylindrocarpon destructans*	Rot disease (Ginseng)	Production of antimicrobial metabolites and/or proteins	Kim et al. (2018)

Table 1 *(Continued)*

Biocontrol agent	Target pathogen	Plant/Disease	Mechanism of action	Reference
Alcaligenes faecalis S18, *Bacillus cereus* S42	*Fusarium oxysporum* f. sp. *lycopersici*	Fusarium wilt (Tomato)	Chitinases and/or proteases	Abdallah et al. (2016)
Bacillus sp.	Drechsleratritici-repentis	Tan spot (Wheat)	-	Larran et al. (2016)
Pseudomonas fluorescens EB69, *Pseudomonas cepacia* EB139, *Pseudomonas* spp. ERG6	Ralstonia solanacearum	Bacterial wilt (Eggplant)	Antifungal/ inhibitory compounds and siderophores	Ramesh and Phadke (2012)
Streptomyces griseus	*Fusarium oxysporum* f. sp. *lycopersici*	Wilt (Tomato)	Chitinases	Anitha and Rabeeth (2009)
Streptomyces spp. AtB42, *Bacillus subtilis* M51 PI	*Pyrenochaeta lycopersici*	Corky root (Tomato)	Antibiotics, antifungal metabolites, and enzyme	Fiume and Fiume (2008)
Bacillus subtilis	*Phytophthora capsica*	Phytophthora blight (Red pepper)	Siderophores, HCN, IAA, phosphatase, and ACC-deaminase	K. J. Lee et al. (2008)
Rhizobium meliloti	*Macrophomina phaseolina*	Charcoal rot (Groundnut)	Siderophores, IAA	Arora et al. (2001)
Pseudomonas chlororaphis PCL1391	*Fusarium oxysporum*	Foot and root rot (Tomato)	Phenazine-1-carboxamide, HCN, chitinases, and proteases	Chin-A-Woeng et al. (1998)

Table 1 *(Continued)*

Biocontrol agent	Target pathogen	Plant/Disease	Mechanism of action	Reference
Chryseobacterium sp. ISE14	Phytophthora capsica	Phytophthora blight (Pepper)	Root colonisation, biofilm formation, and phosphate solubilisation	Sang et al. (2018)
Cladosporium oxysporum PH30409, *Trichoderma koningiopsis* PH30441	*Alternaria panax*, *Fusarium oxysporum*, *Fusarium solani*, *Phoma herbarum*, *Mycocentrospora acerina*	Root rot (*Panax notoginseng*)	Antibiosis, competition, induction of defense response, and mycoparasitism	Zheng et al. (2017)
Trichoderma hamatum, *Bacillus* sp.	Drechslera tritici-repentis	Tan spot (Wheat)	-	Larran et al. (2016)
Streptomyces lavendulae 16R3B	Pythium aphanidermatum	Damping-off (Cucumber)	Chitinases, antibiosis, and competition	Costa et al. (2013)
Trichoderma viride	Pyrenochaeta lycopersici	Corky root (Tomato)	Competition and mycoparasitism antibiotics	Fiume and Fiume (2008)

Note. HCN: Hydrogen cyanide; IAA: Indole acetic acid; ACC: 1-aminocyclopropane-1-carboxylic acid; PRN: Pyrrolnitrin

Application of PGPMs in Biocontrol Activity

In the agricultural sector, microorganisms with several benefits can be valuable and are significant to the bioeconomy in order to fight plant disease. Thus, biocontrol is one of the tools used to manage plant pathogens with low environmental effects (Larran et al., 2016). PGPMs are considered a good biocontrol strategy and play important roles in plant growth-promoting (W. Xu et al., 2019). According to Agrillo et al. (2019), biocontrol agents can be obtained from the extraction and purification of specific compounds isolated from PGPMs. PGPMs have been shown to have several secondary metabolites with antimicrobial properties, resulting in significant antagonistic activity against phytopathogenic fungi (W. Xu et al., 2019). Kim et al. (2018) also indicated that biological metabolites produced from bacteria should generally be considered bio-pesticides. Table 1 shows a few studies establishing pathogen biocontrol using PGPMs and their mechanisms against plant pathogens.

Rhizospheres have been identified as plant growth and health enhancers by supporting mechanisms such as enhanced soil nutrient uptake, phytohormone production and release, and increased plant resistance to environmental stress, which have adverse effects on soil phytopathogens, including fungi, viruses, and nematodes (Prashar et al., 2013). Research done by Abro et al. (2019) also found three promising endophytic fungi, *Penicillium* sp., *Hypocrea* sp., and *Lasiodiplodia theobromae*, which pose as biocontrol agents against wilting of cucumber crops caused by *Fusarium oxysporum* f. sp. *cucumerinum*. These three endophytes effectively reduced the severity of cucumber Fusarium wilt and enhanced cucumber growth (Abro et al., 2019). Murali and Amruthesh (2015) showed that there is a major disease protection of 62% and 58% under greenhouse and field conditions, respectively, in plants pretreated with a conidial suspension of *Penicillium oxalicum*, which is a PGPF against downy mildew disease. Previous research has also reported PGPBs such as *Pseudomonas* (Wicaksono et al., 2018), *Cellulosimicrobium*, and *Bacillus* (Zouari et al., 2016), showing potential against phytopathogenic fungi and/or insects.

CONCLUSION AND FUTURE RECOMMENDATIONS

In this study, plant growth-promoting microorganisms (PGPMs) have shown inherent potential as biocontrol agents against plant pathogens. A great deal of work has already been done in this area. Nevertheless, ongoing efforts are still needed to extend the reliability of these biocontrol products. This is important for the realisation of commercialisation in biocontrol agents because of the high quality and low costs gap between chemical and biological control tools. The complexity of microbial events and their interactions with the environment can be further understood with better screening assays and multi-omics analyses. Such information will pave the way for a generation of markers for the effectiveness

of biocontrol agents. Furthermore, the use of such techniques will allow researchers to measure the influences of plant genotypes and the inherent microbial population on the ecology of a system, apart from suggesting a systematic method to discover novel microorganisms with desired traits.

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CONFLICT OF INTEREST

Authors declare no conflict of interest in this project.

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Diversity of Endophytic Actinomycetes Producing Indole-3-Acetic Acid and *In vitro* Evaluation of Plant Growth-promoting Activity on *Brassica oleracea* L.

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ABSTRACT

The evaluation of endophytic actinomycetes diversity, growth-promoting strain effects on cauliflower seeds germination, and *in vitro* organogenesis are the objectives in this study. Moreover, 15 strains from 125 isolates were determined to produce indole-3-acetic acid (IAA), where majority was obtained from roots (66.67%), followed by from branches (26.67%) and leaves (6.67%). Specifically, *Jatropha* sp. is a plant species with the most endophytic actinomycetes content compared to others. In addition, all endophytic *Streptomyces* strains were screened based on IAA production ability *in vitro* on yeast extract–malt extract broth (YMG) broth medium. The results showed the tendency for one strain with code *Streptomyces* sp. KMR-1E to generate a maximum IAA isolate from *Cinnamomun* sp. plant. Furthermore, the molecular taxonomy and phylogenetic analysis were recreated from 16S rRNA gene sequences, which attributed the KMR-1E to genus *Streptomyces*. Meanwhile, plant growth promotion was evaluated under *in vitro* condition. This exposed the individual ability to enhance the shoot and root length of cauliflowers. The untreated cultures with a strain free agar block was used as control.

Keywords: *Brassica oleracea* L., endophytic actinomycetes, indole-3-acetic acid, plant growth promoter

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INTRODUCTION

Actinomycetes symbolize a group of microbes instrumental in the performance of various beneficial functions, including antimicrobial actions, organic matter fragmentation, alongside mineral solubilization. Also, these bacteria are recognized as producers of plant growth modulators, and have found use in agriculture (Ilic et al., 2007; Suzuki et al., 2000). These organisms are recognized as intrinsic growth regulators, phytohormone synthesizers, as well as groundbreaking resources in plant diseases management (Zhao et al., 2018).

In previous decades, several genera, particularly the endophytic Actinobacteria were separated from the surface-sterilized roots of several plant types. These endophytes, in detail, are bacteria or fungi understood to establish habitats in intracellular, and often intercellular spaces of healthy plant tissue, devoid of the initiation of evident disease symptoms (Maela & Serepa-Dlamini, 2019). Subsequently, the advantageous protection of these organisms from the competitive and demanding soil environment exhibits positive effects on the hosts (Shutsrirung et al., 2014). Moreover, some of the microbe's genetic properties are also shared with the molecular machinery required for plant-specific compound synthesis (Jasim et al., 2014). Also, the existence of endophytic Actinobacteria in the basal organs of healthy crops, including sorghum (de Araújo et al., 2000), banana (Cao et al., 2004), rice (Debananda et al., 2009), as well as the passion fruit (Ali et al., 2017) have been revealed in numerous research articles. Endophytic microbes are worthy of note as growth inducers in various stages of a plant's lifespan including during biological nitrogen fixation (BNF), to facilitate the reduction of dinitrogen (N_2) to ammonia (NH_3), the mobilization of insoluble phosphates, in conjunction with growth enhancement potentials (da Costa et al., 2014; Sharma et al., 2013).

Furthermore, *Streptomyces olivaceoviridis*, *Streptomyces rimosus*, and *Streptomyces viridis* were implicated as members of the unique *Streptomyces* genera of endophytic plants assumed to play a role in IAA synthesis, and subsequently advance plant expansion by enhanced propagation of seeds, alongside root, and shoot extension (El-Tarabily, 2008; Khamna et al., 2010; Tokala et al., 2002). However, data on the yield generated by the *Cinnamomum* sp. and the resultant application in growth promotion is deficient.

Additionally, the cauliflower (*Brassica oleracea* L.) has been cultivated constantly in South Sulawesi for over five decades and is considered an important commercial vegetable. However, increased production indicates a higher use of agricultural chemicals, including manure and insecticides. These chemical applications for extended periods threaten local communities, as well as the environment. Therefore, government policies aim to practice organic agriculture through reduced agrochemical use, and a tactic used involved the application of beneficial microorganisms as inoculants for biocontrol or biofertilizer (Berg, 2009; Malus′a et al., 2012).

Also, several benefits to the bacteria are enhanced through the diversion of host physiological processes by the synthesis of auxins. Moreover, present records demonstrate the strenuous effects of IAA on seed germination, as well as the potential for induction of *Brassica oleracea* L. explant organogenesis by this compound in actinomycetes.

MATERIALS AND METHODS

Sample Collection

All the specimens (roots, branches, and leaves) were collected from a variety plant species in South Sulawesi, Indonesia. Subsequently, the samples were chopped up using a cutter blade, reserved in sterilized plastic bags labelled according to species as well as site sampling, and subsequently returned to the laboratory where bacteria segregation was performed within 48 hours.

Isolation and Selection of Endophytic Actinomycetes

Dirt, debris, as well as surface agents were eliminated from the plant tissues samples by the use of running water. Then, the bacteria were isolated by the reduction of the plant parts into smaller portions (0.2×4 cm^2), and consequently sterilized externally through serial treatment with 70% (v/v) ethanol for 10 minutes. Subsequently, 1% sodium hypochlorite was added for 5 minutes then rinsed four times in sterilized water.

The air-dried specimen was further excised into approximate sizes of 2×3 mm and transplanted onto Starch Casein (SC) agar enhanced with 100 µg/mL nystatin. Moreover, bacteria colonies surrounded the plant tissue at 30°C after an incubation period of 2–3 weeks. These were therefore isolated by the transfer of the clusters onto fresh SC agar plates until purity was indicated by an individual colony. The unadulterated culture was then placed in a 15% sterile glycerol suspension at −80°C to facilitate preservation through an extended period.

Authentication of the Surface Sterilization Protocol

The test procedures were validated by the inoculation of 0.1 mL aliquots of the resultant specimen suspension on SC agar. Subsequently, the samples were incubated at 30°C for 5 days, before microbial growth analysis. The absence of bacterial proliferation confirmed the protocol as effective.

Evaluation for Indole-3-acetic Acid (IAA) Production by Endophytic Isolates

The amount of synthesized hormones was estimated in correspondence with the technique proposed by Bano and Musarrat (2003). This was implemented by the cultivation of the agar block (6 mm in diameter) of the bacterial colonies on yeast extract–malt extract broth (YMG) (g/L) (made up of yeast extract 16 g, malt extract 10 g, and glucose 4 g), alongside a final incubation at 35 °C for a period of seven days. Then, the culture was transferred into 5 mL YMG broth inclusive of 2 mg/mL L-tryptophan, before the final incubation

and concurrent agitation at 35°C and 125 rpm for 7 days, respectively.

The cells were consequently harvested by using a centrifuge at 20,328 x *g* for 15 minutes, and 1 mL of the upper layer of the product combined with 2 mL of Salkowski reagent (1 mL of 0.5 M $FeCl_3$, in 49 mL of 35% w/v $HClO_4$), with the resulting solution incubated at 30 °C for 25 minutes in an environment without light. Subsequently, conversion of the color to a pink hue was suggestive of a positive test. The optical density (OD) was then ascertained with the employment of a spectrophotometer with a wavelength of 535 nm, and the resultant level of IAA produced appraised against the standard.

Preliminary Phenotypic Characterization of IAA Producing Strain

A phenotypic characterization was performed on all strains producing endophytic IAA using the International Streptomyces Project (ISP) medium. Therefore, the morphological and cultural properties, including the spore mass color, existence of aerial hypha, diffusible pigments color, unique reverse colony color, as well as spore chain morphology were evaluated (Cao et al., 2004).

Expulsion and Identification of IAA

This detection process involved the use of isolates assumed to produce the highest IAA. The area above this culture was then obtained using a centrifuge at 20,328 x *g* for 15 minutes after fermentation on YMG broth media supplemented with 2 mg/mL L-tryptophan at 35 °C and 125 rpm for 7 days. Also, IAA extraction was conducted based on the procedure from Ahmad et al. (2005). This required acidifying the culture supernatant to pH 2.5 with HCl, and the supernatant culture: ethyl acetate (1:2 v/v) was subsequently adopted. The confirmation of isolates determined to produce this acid was conducted using thin layer chromatography (TLC) methods. This method was further executed with 25 µL ethyl acetate macerate stained on TLC plates (silica gel GF_{254}, thickness 0.25 mm, Merck, Germany) and developed using ethyl acetate: chloroform: formic acid (55:35:10 v/v) as the mobile phase. Furthermore, UV light (254 nm) was used to confirm the points with similar retention factor (R_f) values as the original pure IAA chemical after Ehmann's reagent was sprayed on the LC plates (Vikram, 2011).

Optimization of IAA Production Condition

The IAA production process was improved by evaluating the impact of incubation period on the culture temperature and pH condition. Therefore, similar medium and conditions as described above were used to culture the isolate. Furthermore, the incubation period effect was examined over a 10-day period.

Effect of Soaking on Seed Germination

The cauliflower seeds were subjected to surface sterilization for 10 minutes, using 10% (w/v) NaOCl. This was followed by

a thorough wash with sterile distillated water, and air drying, before soaking in the KMR-1E strain culture filtrate (grown in YMG broth medium broth amended with L-tryptophan 2 mg/mL, incubated for 7 days at 30°C). Furthermore, the authentic IAA (Merck) was immersed in the same sterile distillated water (0.5 µg/mL) and applied as the study control. Therefore, a tentative therapy was conducted over the agar block of KMR1 strain (grown on YMG agar medium modified L-tryptophan).

The seeds were then cultured on the water agar media (5% w/v) placed within a Petri dish (9 cm in diameter), and arranged vertically in the growth chamber, in an attempt to facilitate seed maturation with no restrictions. Subsequently, the extent of growth was evaluated after 10 days of germination, based on number of roots, fresh weight of plant, primary root, and shoot length.

Assessment of Plant Growth Promotion in Cauliflower Tissue Culture Treated with Specific Endophytic Actinomycetes

The KMR-1E strain growth promotion capacity was assessed *in vitro* in a bottle chamber (6 cm in diameter and 20 cm height). This was followed by using tap water to wash the cauliflower stalk and remove particles as well as other agents. The representative cauliflower (2 × 2.5 cm) obtained per explant was purified through 10 minutes of continuous submersion in NaOCl 10% (w/v) and consequently soaked in an autoclaved distilled water four times. Subsequently, the explants were finally air dried after cutting into thin blocks (2 × 10 mm) and placed without or with IAA on the basal MS medium. This vehicle had an adjusted pH of 5.8 prior to autoclaving. Meanwhile, the IAA stock solution was prepared with 100 mL purified water, and filtered with a 0.2 µm sterile membrane filter.

The control (K0) sample encompasses the flowers branches of cauliflower explant yield cultivated on Murashige and Skoog (MS) primary medium with 1% sucrose. Also, different treatments, including the explant grown in this agent, and free strain YMG agar block medium modified L-tryptophan (NS) were evaluated, and consequently transferred at a ratio of 1 to 3 per explant. In addition, YMG medium agar block amended L-tryptophan with KMR1 strain (ST) was used in one of the experimental treatments, while the other involved genuine IAA (PC) altered to 0.15, 0.2, and 0.25 µg/mL, respectively. Figure 1 shows the test reserved within the conditions of 24 °C, 24 h light, and 60% mm for the cultivated plant part. Therefore, measurements were obtained all parameter data after a 3-week culture period. The three treatments were conducted using a completely randomized design.

Molecular Identification of Selected Endophytic Actinomycetes by 16S rDNA Sequence Analysis

The selected KMR-1E strain cells were cultured in YMG for 4 days. Therefore, the DNA genome was prepared with minor modification, according to Badji

et al. (2006). In addition, two primers were applied during the amplification of 16S rDNA, including: 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3'. Also, through an Invitrogen™ Kit, polymerase chain reaction (PCR) was adopted for gene sequence amplification, and the final reaction mixture volume (50 µL) comprised of 50 ng template DNA; 10 *p*mol primer concentration; PCR master mix containing *Taq* DNA polymerase, dNTPs, Tris-HCl, $MgCl_2$ stabilizer; as well as tracking dye applied based on the manufacturer's instructions. The amplification process was conducted on a thermal cycler (Gene Cycler™, Bio-Rad) by exposing sample to an initial denaturation step for 5 minutes, with temperature set at 96 °C. Subsequently, 30 amplification cycles of 95 °C for 1.5 minutes, 55.5 °C for 1 minute, 72 °C for 5 minutes, and 72 °C for 5 minutes was conducted. Therefore, electrophoresis was adopted for gene detection using 1.5% (w/v) agarose gels stained with ethidium bromide. In addition, using BLAST NCBI search (http://blast.ncbi.nlm.gov/), the 16S rRNA gene sequence in KMR-1E variants was subjected to similarity analysis. Subsequently, an evolutionary tree was suggested based neighbor-joining (Saitou & Nei, 1987), while phylogenetic analysis and multiple alignments involved the Clustal X program (Thompson et al., 1994). The tree topology was evaluated based on 1,000 replicates, and using the bootstrap analysis (Felsenstein, 1985).

Statistical Analysis

Data were subjected to ANOVA using SPSS software version 16.0 for Windows. Statistical differences between means were compared using least significant difference (LSD) test at $p = 0.05$.

RESULTS AND DISCUSSION

Diversity of the Endophytic Strain

The various plant tissue possessed a total of 125 endophytic actinomycetes strains, and

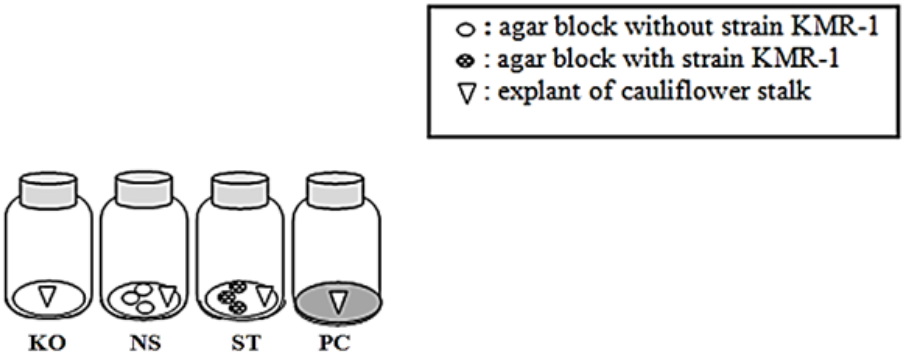


Figure 1. Schematic of *in vitro* evaluation of growth promoting substances produced by endophytic actinomycetes on cauliflower stalk plant growth promotion response

15 were selected for further studies. Table 1 illustrates the negative bacteria growth on SC agar medium in the representative samples from every crop treated with the surface sterilization protocol. This proves the elimination process for this organism was effective using this method. Furthermore, from the isolates screened for endophytic plant, the highest and lowest number of strains were obtained from the roots and leaves where n = 64.4% and 18.4%, respectively. These were discovered to produce IAA. The majority of isolates were separated from roots (66.67%), branches (26.67%), and leaves (6.67%). In addition, most of the bacteria were obtained from *Jatropha* sp. and the predominant plant had at least one isolate yield, therefore the organism is able to colonize different parts of various tissues.

The isolation of microbe in a culture medium is a simple method for assessing the bacterial communities (Andreote et al., 2009), although this is an underestimation in terms of diversity relating to the microbial populations. This is because bacterial identification is significantly influenced by the growth of selected specific culture medium. Also, microbial populations vary for different vegetation and species inhabit. The populations of microbes differ depending on the locations or climatic conditions even in similar plant species (Nair & Padmavathy, 2014). Moreover, colony appearances, including substrate hyphae and spore mass color on ISP Medium No. 3 displayed the

Table 1

The occurrences of endophytic actinomycetes strain from different plant species

Plant species	Plant tissue of endophytic strain sources			No. of endophytic strain
	Roots	Stem barks	Leaves	
Cinnamomum sp.	12 (2)	1	1	14
Piper sp.	5 (1)	2 (1)	4	11
Ficus	14 (1)	4	5	23
Hibiscus sp.	6	2 (1)	1	9
Areca sp.	7 (1)	1	1	9
Unidentified fern	11 (1)	3	4	18
Manihot sp.	9 (1)	1	4	14
Jatropha sp.	17 (3)	7 (2)	3 (1)	27
Total	83 (10)	21 (4)	23 (1)	125
Percentage	64.4% (66.67%)	16.8% (26.67%)	18.4% (6.67%)	

Note. *Value in brackets indicates the number of endophytic actinomycetes strain IAA producing

isolates in form of two different groups. These included the *Streptomyces*-like strain distinguished by an abundant aerial mycelium with powdery spores and the non-*Streptomyces* variant marked by red, orange, and brown to black slimy colonies. The *Streptomyces*-like strain represented 66.67% of the endophytic species, majorly the S of spore chain type. According to Sardi et al. (1992), 499 endophytic actinomycetes were separated from various crops, with approximately 96% *Streptomyces* and 0.2% *Streptosporangium*. The non-*Streptomyces*-like strain was discovered to colonize roots in this study as noted earlier by Goudjal et al. (2013).

Screening of IAA Production of Endophytic Strain

The isolates screening for the presence of beneficial traits indicated 15 strains were discovered to be the producers of IAA. This acid generation has a high variability range of 17.65 to 86.94 µg/mL for *Streptomyces*-like strain and a value of 21.41 to 68.12 µg/mL for non-*Streptomyces*-like strain. Furthermore, 8 plants of endophytic actinomycetes were discovered with the potential to generate this acid in the present study. Table 2 demonstrates the high production ability of two variants termed KMR-1E and KMR-11. This was the most significant amongst all other genera, with a value of 86.94 µg/mL. Also, IAA was determined to be the main auxin in plants, due to the vital function possessed by this acid in the first processes of lateral and adventitious root formation and elongation (Idris et al., 2007). The presence of microorganism colonies in plant tissue has a vital role in vegetation. According to Shutsrirung et al. (2014), IAA produced by *Streptomyces* spp. was ranged between 1.05 to 60.95 µg/mL and 11 to 54 µg/mL. This study discovered a high variation level in the auxin production by *Streptomyces* with the exception of *Streptomyces viridis* CMU-H009 at a value of 143.95 µg/mL (Khamna et al., 2010).

Table 2

Morphological characteristic and estimation of IAA production of endophytic Actinomycetes

Groups	Plant sources	Diffusible pigment	Spore-chain type*	Group of genera	Concentration of IAA (µg/mL)
KMR-1E	*Cinnamomum* sp.	-	F	Streptomyces	86.94±0.71
KMR-2	*Cinnamomum* sp.	-	RF	Streptomyces	38.00±0.67
KMR-4a	*Piper* sp.	-	S	*Streptomyces*	64.59±0.69
KMR-4b	*Piper* sp.	-	R	*Streptomyces*	86.29±0.79
KMR-11	*Ficus* sp.	Yellow	RA	*Streptomyces*	86.92±0.77
KMR-12	*Hibiscus* sp.	-	RF	*Streptomyces*	65.18±0.42
KMR-15	*Areca* sp.	-	S	*Streptomyces*	36.59±0.57

Table 2 *(Continued)*

Groups	Plant sources	Diffusible pigment	Spore-chain type*	Group of genera	Concentration of IAA (μg/mL)
KMR-22	Unidentified fern	-	-	Non-*Streptomyces*	21.41±0.31
KMR-23	*Manihot* sp.	Brown	-	Non-*Streptomyces*	68.12±0.78
KMR-26a	*Jatropha* sp.	-	S	*Streptomyces*	17.65±0.34
KMR-26b$_1$	*Jatropha* sp.	-	S	*Streptomyces*	19.06±0.21
KMR- 26b$_2$	*Jatropha* sp.	-	-	Non-*Streptomyces*	61.06±0.59
KMR- 26c	*Jatropha* sp.	-	S	*Streptomyces*	44.00±0.62
KMR- 26d	*Jatropha* sp.	-	-	Non-*Streptomyces*	27.53±0.33
KMR- 26e	*Jatropha* sp.	-	-	Non-*Streptomyces*	66.94±0.58

Note. *RA = Rectiaculiaperti; RF = Rectiflexibiles; S = Spiral. IAA was estimated 7 days after the endophytic actinomycete growth in YMG broth medium supplemented 2 mg/mL of L-tryptophan with absorbance at 540 nm

Detection and Optimization of IAA Production by KMR1 Strain

The detection and comparison of IAA-producing strain with standard auxins was performed using TLC chromatograms. Furthermore, pink color spot of ethyl acetate extract at R_f 0.6, correspond to the authentic compound, therefore IAA-producing organism is a potential source for the chemical. Meanwhile, this technique is important for the qualitative or semi-qualitative determination detection of the acid. This method assured the hormone presence in the unrefined top layers of these samples of bacterial cultures. The Salkowski reagent reacted with the acid but not with L-tryptophan and Na-acetyl-L-tryptophan (Vaghasiat et al., 2011). Figure 2 presents the effects of incubation periods toward the hormone production of KMR-1E strain. The results showed this process commenced after 24 hours, and gradually increased after 7 days of incubation with a reduction on the 8th day. This impact was studied to acquire knowledge on the optimal culture condition for the highest producer termed KMR-1E. Also, adjustments were made to the culture media pH, temperature, and L-tryptophan concentration with reference to previous studies. The maximum yield was obtained after a 7-day incubation period, as shown in the results time exponent. This reduction occurs as a result of oxidase and peroxidase activity as indicated in the *Rhizobium* sp. from *Cajanus cajan* (Datta & Basu, 2000). This is congruent with the agreement in a research on *Streptomyces* sp. PT2 (Goudjal et al., 2013).

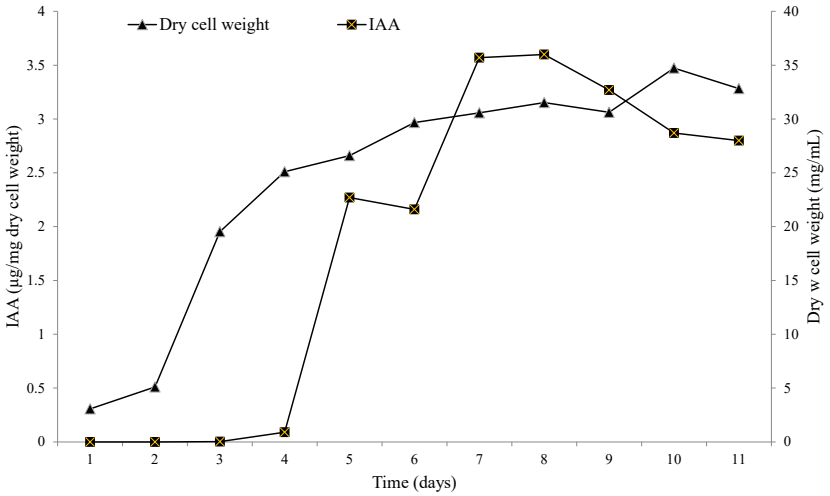


Figure 2. Time course of indole-3-acetic acid (IAA) production by strain *Streptomyces* KMR-1E on YMG broth (amended L-tryptophan 2 mg/mL; pH 7±.05) at temperature 35°C for different incubation periods. In addition, each value was determined as the mean of 3 replicates

Effect of Presoaking Periods and IAA Treatments on Seed Germination and Root Elongation

Table 3 shows a significant improvement in the germination of inoculated cauliflower seeds induced by the growth modulator of the KMR-1E strain against an uninoculated control, examined over 10 days according to the test conditions. Furthermore, an agar block of medium containing the strain alongside IAA standard exhibited greater limb lengths and biomass, unlike the pre-soaked seed untreated with the crude supernatant of the KMR-1E strain (control). Meanwhile, important variations were not noted between specimens processed with IAA and an agar block of YMG without L-tryptophan, in terms of percentage root and shoot lengths, higher biomass, as well as the number of adventitious roots.

The samples impregnated with IAA, YMG agar slab alongside the strain modified with L-tryptophan demonstrated substantial distinctions when assessed against the control, as well as the aforementioned pre-soaked seed sample. Therefore, the seed treatment was revealed to have affected the root and shoot expansion as shown in Figure 3. Subsequently, the ensuing outcome proved the immersion of the seeds for 5 hours in standard IAA and agar block YMG medium amended with L-tryptophan as the major responsive assay. This outcome approved a former research performed with sugar beets (Shi et al., 2009), where considerable growth of the seedlings was observed. Several researches corroborated the ability of endophytic actinomycetes to synthesize IAA, and consequently improve plant growth by intensifying

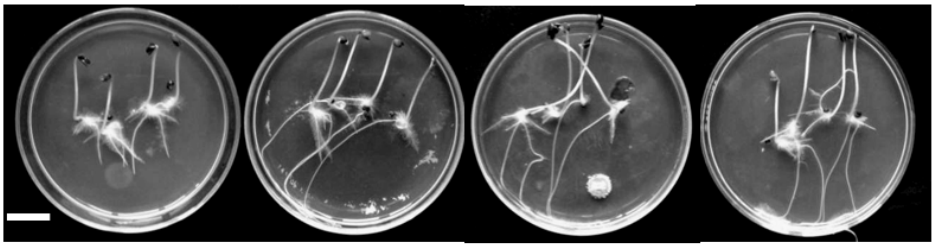
plant cultivation. The improvement of tomato seeds with the culture top layer containing the unadulterated IAA was also observed. Also, previous documentation described the advanced seed germination and root extension produced in *Ochetophilia trinervis* by IAA-generating endophytic actinomycetes (Solans et al., 2011). Also, the *Streptomyces* sp. isolated from *Centella asiatica* possessed the potential to enhance the earlier stated characteristics (Dochhil et al., 2013).

Table 3

Effects of endophytic actinomycetes strain on the growth of cauliflower seedling

Treatment	Fresh primary root length (cm)*	Fresh shoot length (cm)*	No of adventive roots*	Fresh plant biomass weight (g)*
(A) Control	2.025[a]	2.550[a]	2.750[a]	0.046[a]
(B) YMG broth medium with KMR-1E strain and amended L-tryptophan	6.950[b]	4.700[b]	5.250[b]	0.061[ab]
(C) YMG solid medium with KMR-1E strain and supplied L-tryptophan	7.720[b]	4.950[ab]	5.750[b]	0.079[b]
(D) IAA authentic standard	7.550[b]	4.900[ab]	6.100[b]	0.073[b]

Note. *It was measured after 10 days of seedling. Means followed by the same lowercase letter within the same column were not significantly different based on LSD test (p = 0.05). There were 3 replications per treatment (4 plants per replication)



A B C D

Figure 3. Early development of cauliflower (*Brassica oleracea* L.) seedling. These seeds were surface sterilized and placed on water agar (5%) for germination at 25°C in a plant growth chamber for 10 days. The images showed the representative photographs of the cauliflower seedling, and each scale bar represents 2 cm

Note.

A. *Brassica* seeds were applied with sterile aquadest as seed soaking treatments
B. *Brassica* seeds were soaked with YMG broth medium with KMR-1E strain
C. Media were applied as block agar YMG medium with KMR-1E strain placed on surface of growth media
D. *Brassica* seeds were soaked with IAA pure chemical (0.5 µg/mL)

The Organogenesis of the Cauliflower Explants through *In vitro* Procedures

The stalk explants with dimensions of 2 × 2.5 cm were transferred to a basal MS medium, and the length of the plant limbs, alongside the induction of adventitious roots were observed across all the samples after fourteen days of culture. However, the optimal response was observed for the cultures comprising of the strain adjusted with L-tryptophan and IAA, where sizeable variations in branches and roots sizes, as well as the absence of alterations in the number of leaf regeneration, in contrast to the control and the sample without strain ($p < 0.05$), were displayed. Therefore, species producing IAA from media enriched with L-tryptophan displayed greater potentials for shoots and adventitious roots height extension, as well as biomass weight enhancement in contrast with the control (Figures 4-5), as indicated by the results. Consequently, the potential of IAA-producing endophytic actinomycetes to induce the organs of *B. oleracea* L. explants was demonstrated. Despite the tissue culture on MS basal medium and treatment with YMG agar block, the media provided insignificant responses compared with the control. The medium treated with strain and IAA exhibited better outcomes of explant regrowth. Furthermore, cauliflower regeneration was influenced by several factors, including the media components (Kaur et al., 2006; Ovesna et al., 1993), growth regulators (Hoque, 2010), culture conditions as well as explant types (Kerlley et al., 2012). In general, the addition of auxin and bacterial metabolites were observed to appreciably improve organogenesis, especially of the plant limbs and roots. Also, the utilization of actinobacterial cultures for inoculation also significantly improved growth and development, specifically of the plant limbs, and biomass (Jog et al., 2014).

This experiment has also demonstrated the comparable *in vitro* shoot and root induction potential of IAA-producing endophytic actinomycetes to the commercially available phytohormone. The results indicate the inability for minimum concentrations in YMG medium agar block to demonstrate significant organogenesis response in contrast with IAA standards for all parameter except number of roots.

Characteristics of *Streptomyces* sp. KMR-1E Strain

Figure 6 shows the reconstruction of molecular taxonomy and phylogenetic analysis based on 16S rRNA gene sequences. This phenomenon prompted the strain designation to genus *Streptomyces*. KMR-1E strain shared 97% similarity with *Streptomyces tendae* NBRC 12822, and thus assigned as a closely related species to *Streptomyces albogriseolus* DSM 40003 and *Streptomyces violaceolatus* NBRC 13101. In addition, there was a spiral chain spore formation with KMR-1E strain. Meanwhile, the *S. tendae* retinaculiaperti or spiral on salts starch agar. *S. tendae* demonstrated a massive gray aerial color on yeast lactose, salts-starch agar, and oatmeal, while KMR-1E strain showed a pure white coloration, as observed in Table 4. The reverse side

of a colony is yellow, while the KMR-1E strain showed a sand yellow coloration. This particularly utilized glucose, raffinose, inositol, sucrose, and D-mannitol while the *S. tendae* showed no growth signal on raffinose (Whitman et al., 2012).

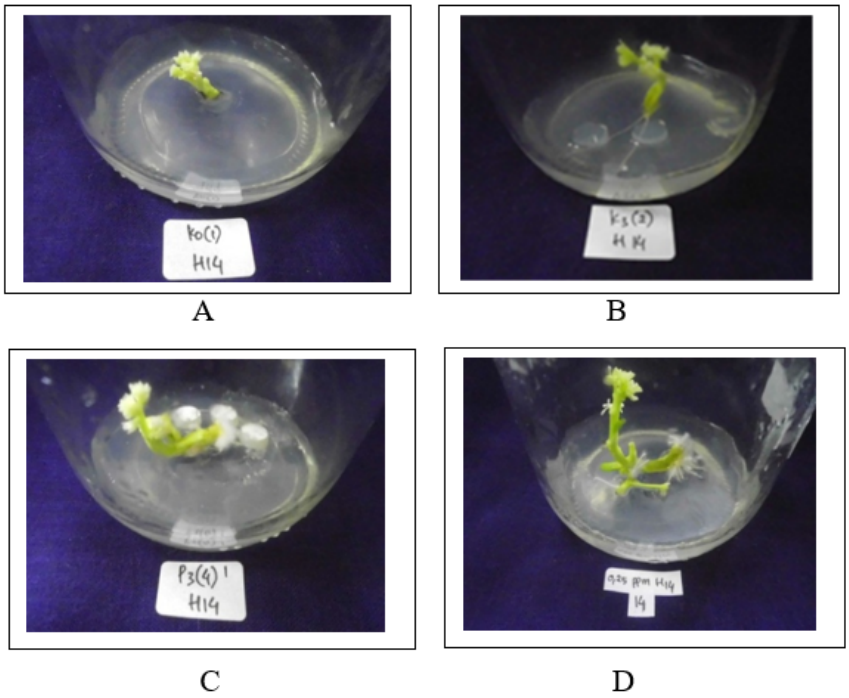


Figure 4. Effect of strain KMR-1E agar block on the organogenesis of cauliflower stalk after a 14-day culture period. The flower branches were cultured on Murashige and Skoog (MS) basal medium supplemented with 1% sucrose and consequently used as (A) control (K0) then MS basal medium alongside agar block of YMG medium, (B) without strain and amended L-tryptophan (NS), (C) characterized by amended L-tryptophan with KMR-1E strain (ST), (D) treated by authentic IAA (PC)

Table 4

Cultural characteristics of KMR-1E strain on different culture mediums

Agar medium	Aerial mycelium	Substrate mycelium	Growth
ISP1	Pure white	Brown beige	Good growth
ISP2	Pure white	Sand yellow	Moderate growth
ISP3	No growth	No growth	No growth
ISP4	Ochre brown	Orange, brown	Good growth
ISP5	Cream	Cream	Moderate growth
ISP6	Pure white	Sand yellow	Good growth

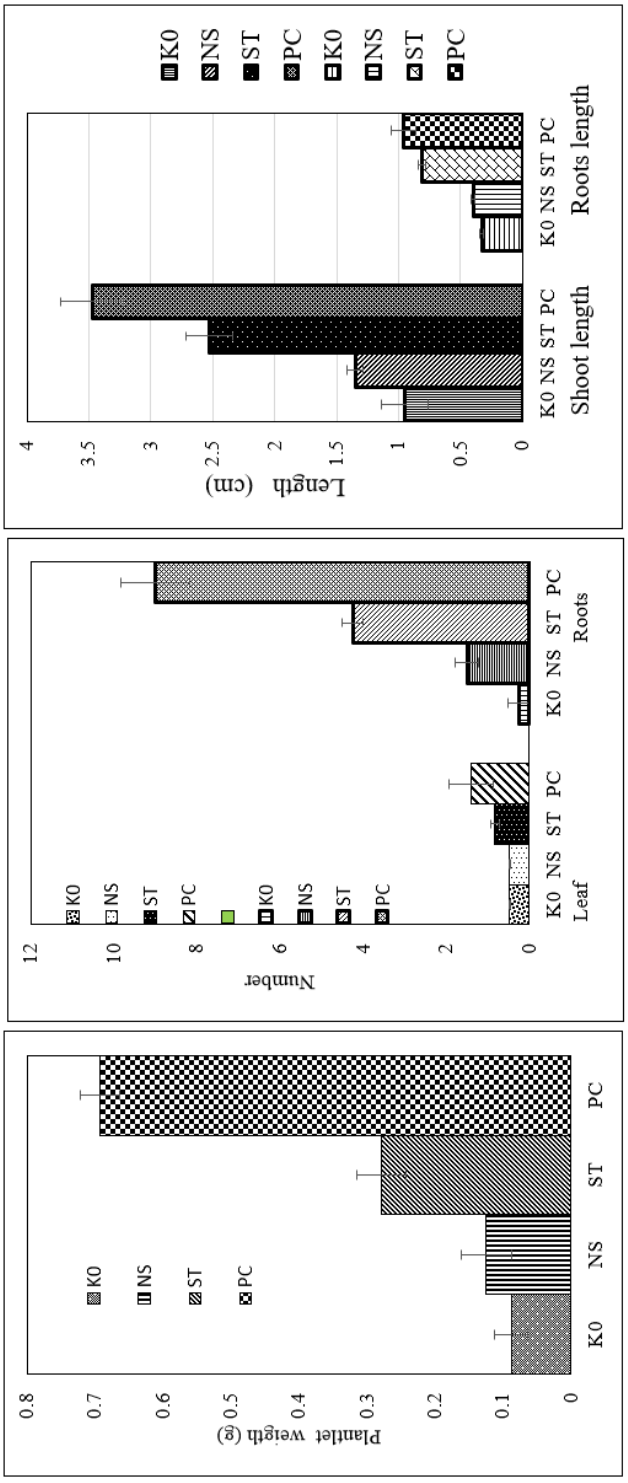


Figure 5. Effect of *in vitro* organogenesis of cauliflower stalk explant on (A) plantlet weight, (B) number of leaf and adventive roots, and (C) shoot and roots length. *Note*. K0 = Control; NS = Agar block medium modified L-tryptophan; ST = Agar block amended L-tryptophan with KMR1 strain; PC = Experimental treatments with genuine IAA

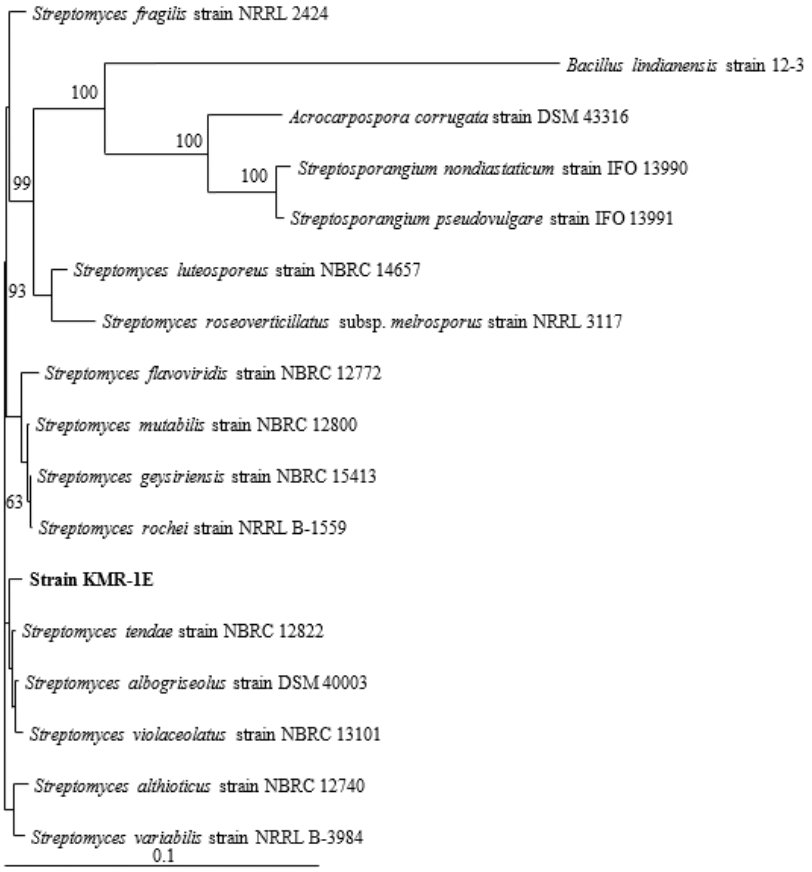


Figure 6. Neighbor-joining phylogenetic tree inferred from 16S rRNA gene sequences. This shows the relationship between endophytic bacteria KMR-1E strain and related genera. The bootstrap values were further expressed as percentages of 1,000 replications, and those ≥ 50% are expressed at branch points. Furthermore, the score bar denotes 1 nucleotide substitution per 100 nucleotides

CONCLUSION

Fifteen (15) isolates of actinomycetes recovered from plant tissue could provide interesting sources of endophytic strains producing indole-3-acetic acid. The work demonstrates the potential of strains *Streptomyces* sp. KMR-1E showed the indole-3-acetic acid produced by endophytic actinomycetes isolates of *Cinnamomun* sp. plant. The strain has the potential to promote seedling germination as well as induce shoot and root *in vitro* organogenesis in cauliflower stalk explant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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ABSTRACT

High-quality RNA is an important genetic study as it has minimal contaminants that can affect gene discovery including degraded RNAs, chemical, and biological residues. Hence, it is a prerequisite for genetic analysis using Next Generation Sequencing (NGS) for accurate and reliable data mining. Despite its importance, extracting high-quality RNA from different samples is often a challenge, as every tissue has a different biochemical composition, thus requiring different protocols. This paper reports protocols for the extraction of high-quality RNA from two type of pineapple tissues, which are thickly lignified hard tissue (tillers, inflorescence, flowers) and watery soft tissue (mature fruit, ripe fruit, and overripe fruit) via modified Kim and Hamada (2005) method. Total RNA was extracted in all six tissues, which showed two distinctive 25S and 18S band on agarose gel. The total RNA in this study was considered high-quality as the minimum concentration was 50 ng/µl, the absorbance ratio (A_{260}:A_{280}) was more than 1.8 and RNA integrity number (RIN) was greater than 7. The obtained results showed that the modified Kim and Hamada (2005) method was effective in extracting high-quality RNA from the challenging MD2 pineapple tissue, which is suitable for subsequent molecular analysis, including the highly sensitive NGS.

Keywords: MD2 pineapple, Next Generation Sequencing, RIN number, RNA extraction

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INTRODUCTION

Next-Generation Sequencing (NGS) is one of the most utilised platforms providing reliable large-scale data within a short time. It facilitates the research of various plant mechanisms and system (Godden et al., 2012; Soltis et al., 2013). Indeed, numerous molecules including DNA, mRNA, small RNA, degraded RNA, long non-coding RNA, exons, and DNA fragments are now being sequenced using various NGS platforms. In the highly sensitive RNA-sequencing or transcriptomic analysis, the quality of the data obtained from these NGS platforms ultimately depends on the quality of the starting materials. However, obtaining high-quality RNA from plants that meets requirement for RNA-sequencing remains a challenge.

Many studies have demonstrated the successful isolation of total RNA from biological samples including human, animal, plant, bacteria, fungus, and viruses (Cox, 1968; Holmes et al., 2014; Kałużna et al., 2016). RNA extraction is the process of isolating purified total RNA from the living source samples with minimal degradation and contamination. However, no standardized protocol can be used due to the different composition of various samples that demand different extraction protocols, especially in high secondary metabolite samples as phenolic compounds readily oxidized to form quinones which bind to nucleic acids, preventing extraction of high-quality RNA (Ghangal et al., 2009). Therefore, the extraction protocol should be modified depending on the sample for the extraction of high-quality RNA (Atshan et al., 2012; D. Li et al., 2009; Liu et al., 2018).

Pineapple (*Ananas comosus*) is an important commercial fruit (Hossain et al., 2015), as well as a model organism for non-climacteric ripening, rich in polysaccharides, polyphenols, water content, lignin, and fibre (Liu et al., 2018). This study reports a protocol suitable for the extraction of high-quality RNA from six pineapple tissues including the tiller, inflorescent, flower, mature fruit, ripe fruit, and overripe fruit. The protocol was modified from Kim and Hamada (2005), which reported DNA and RNA extraction method in sweet potato. The method was equipped to extract total RNA from several tissues of MD2 pineapple which was unsuccessful perhaps due to different tissue composition, resulting in few modifications to obtain high-quality RNA suitable for RNA-based analyses using various platforms utilising NGS technology. Total RNA can be deemed as high-quality if it fits the minimum criteria of NGS requirement which are (a) minimum concentration of 50 ng/µl, (b) absorbance ratio (A_{260}:A_{280}) of more than 1.8, and (c) RNA integrity number (RIN) value greater than 7. In addition, total RNA of high quality can be used for different analytical techniques, including reverse transcription polymerase chain reaction (RT-PCR), real-time fluorescent quantitation polymerase chain reaction (qPCR), and microarray analysis (Ma et al., 2015).

MATERIALS AND METHODS

Plant Materials and Apparatus Treatment

Six MD2 pineapple tissues were used in this study and three of them were hard tissues collected from the tiller, inflorescence, and flower, while the other three were soft tissues collected from mature fruit (fruit at ripening index 1), ripe fruit (fruit at ripening index 4), and overripe fruit (fruit at ripening index 7). The level of maturity of the fruits are measured based on the colour of the skin of the pineapple (Bakar et al., 2013). The samples were collected as per Sabah Department of Agriculture guidance at a farm located in Kampong Poring, Inanam, Sabah, Malaysia, in which the tissues were transported in clean plastic bag. Upon arrival, each tissue was rinsed using sterile distilled water and cut into smaller pieces before placing inside clean sterile plastic. The tissues were then stored at -80°C before extraction. RNAse pre-treatment of apparatus and plastic ware was also done by dipping in diethyl pyrocarbonate (DEPC) solution overnight, before sterilizing via autoclave.

Extraction of Total RNA from Hard and Soft Tissue

Hard Tissues (Tillers, Inflorescence, and Flowers). Total RNA from hard tissues including tillers, inflorescence, and flowers was extracted using a protocol modified from Kim and Hamada (2005). RNA was extracted from approximately 3 g of the frozen sample which was powdered using a chilled mortar and pestle. The samples were transferred into a fresh tube containing 3 mL of lysis buffer consisting of 1.4 M NaCl, 20 mM EDTA, 100 mM Tris (pH 8.0), 2% CTAB, and 2% β-mercaptoethanol, then half volume (v/v) of 6 M sodium chloride (saturated NaCl) was added and incubated at 65°C for 10 minutes. The samples were centrifuged at 16,600 x *g* for 12 minutes at 4°C to collect the supernatants, which were then mixed with an equal volume of phenol: chloroform: isoamyl alcohol (125:24:1; pH~4.5). The samples were centrifuged again at 16,600 x *g* for 12 minutes at 4°C to collect the uppermost aqueous layer, which was transferred into a fresh tube before adding an equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 16,600 x *g* for 12 minutes at 4°C. The uppermost aqueous layer was again collected, mixed with an equal volume of absolute ethanol and incubated overnight at -20°C. The RNA precipitate was centrifuged at 16,600 x *g* for 12 minutes at 4°C, the supernatant was discarded, and the RNA pellet obtained was washed twice with 70% ethanol. The RNA pellet was air-dried in a vacuum concentrator before resuspension in 30 µL DEPC-treated water.

Soft Tissues (Mature, Ripe, and Overripe Fruits). For the extraction of total RNA from soft tissues including mature, ripe, and overripe fruits, the same protocol for the extraction of hard tissues was used with minor modifications. First,

the extraction was performed using an extraction buffer containing 400 mM NaCl, 20 mM EDTA, 10 mM Tris-HCl, 1% SDS, and 2% β-mercaptoethanol. Secondly, the soft tissue samples were not incubated at 65°C for 10 minutes.

Modification from Kim and Hamada Protocol

The pineapple tissues were extracted using a protocol modified from Kim and Hamada (2005), from now on referred to as the KH protocol. The five major procedures performed for the extraction of soft and hard tissues of pineapple are as follows:

a. The initial buffer in the KH protocol contained 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris/HCl (pH 9.5), 2% CTAB, and 1% β-mercaptoethanol. This was modified to contain 2% β-mercaptoethanol and Tris/HCl was replaced with Tris (pH 8.0) for the extraction of hard tissues and soft tissues, and 2% CTAB was replaced with 1% SDS for extraction of soft tissues.

b. Instead of adding saturated LiCl (10 M) after the addition of the lysis buffer in the KH protocol, saturated NaCl (6 M) was added for the extraction of hard and soft tissues.

c. The incubation step at 65°C for 10 minutes was omitted for the extraction of soft tissue samples.

d. The KH protocol involved two extractions using a C:I (24:1) mixture, in this study, the first extraction was performed using P:C:I (25:24:1), while C:I (24:1) was used for the second extraction of hard and soft tissues.

e. The RNA samples of the pineapple tissues were precipitated with absolute ethanol rather than LiCl (10 M) in this study.

RNA Concentration, Purity, and Integrity Analyses

Total RNA for each sample was visualised by 1.2% agarose gel electrophoresis with 1X TAE buffer. The total RNA concentration and purity (absorbance A_{260}:A_{280}) were quantified using a NanoDrop™ Spectrophotometer. The RIN was then calculated using an Agilent 2100 Bioanalyzer system and Agilent RNA 6000 Pico Kit.

RESULTS

The six pineapple tissues, namely tillers, inflorescent, flower, mature fruit, ripen fruit, and overripe fruit (Figure 1) were first categorised as hard or soft tissues. Hard tissues (tillers, inflorescence, and flowers) typically are composed of a thick lignified wall (Yahia & Carrillo-López, 2018), while soft tissues (fruit) contain 80% water (Cordenunsi et al., 2010).

The presence of 25S and 18S ribosomal RNA (rRNA) was observed in the RNA extracted from all tissues using the modified protocols (Figure 2) and was consistent for all replicate samples (n = 3). The quality of the extracted RNA was verified using an automated gel electrophoresis system, demonstrating a RIN ranging from 6.3 to 9.5, with a total RNA concentration between

134.3 ng/µl to 1074.6 ng/µl (Figure 3). The gel images also confirmed the RNA integrity. The concentration, purity ratio and RIN for each tissue with respective biological replicate are summarized in Table 1.

Table 1

Concentration, purity ratio and RIN value for each pineapple tissues including biological replicate

Tissue	Biological replicate	Concentration (ng/µl)	A260/280	RIN no.
Tiller	1	442.6	2.01	7.7
	2	308.8	2.12	9.5
	3	338.2	2.13	7.7
Inflorescence	1	678.6	2.05	8.2
	2	528.1	2.08	8.7
	3	673.1	2.08	7.3
Flower	1	1074.6	2.16	7.2
	2	709.3	2.08	7.6
	3	449.8	2.08	7.2
Mature fruit (Fruit at ripening index 1)	1	309.7	2.11	7.4
	2	137.7	1.87	7.2
	3	533.0	2.03	7.8
Ripe fruit (Fruit at ripening index 4)	1	434.5	2.01	7.8
	2	134.3	2.09	7.1
	3	495.1	2.05	9.0
Overripe fruit (Fruit at ripening index 7)	1	243.7	1.96	7.9
	2	200.6	2.12	9.1
	3	205.4	2.13	8.3

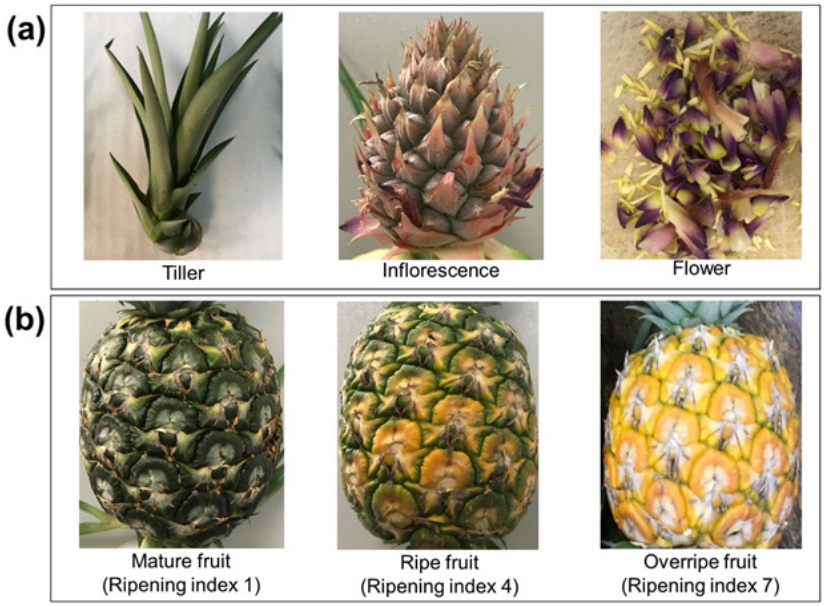

Figure 1. Samples of MD2 pineapple used for RNA extraction. (a) Hard tissue samples of tillers, inflorescence, and flowers; (b) soft tissue samples of mature fruit (at ripening index 1), ripe fruit (at ripening index 4), and overripe fruit (at ripening index 7)

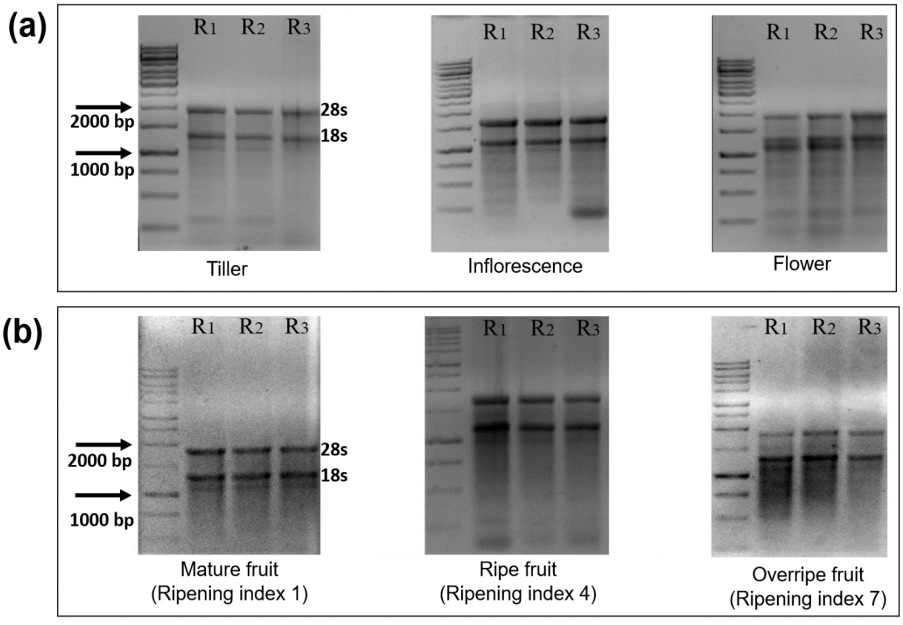

Figure 2. Presence of intact 25S and 18S rRNA in the RNA extracted from pineapple tissues with three biological replicates (R1, R2, and R3), run with '0' GeneRuler™ 1kb DNA ladder. (a) Hard tissue samples of tillers, inflorescence, and flowers; (b) soft tissue samples of mature fruit (at ripening index 1), ripe fruit (at ripening index 4), and overripe fruit (at ripening index 7)

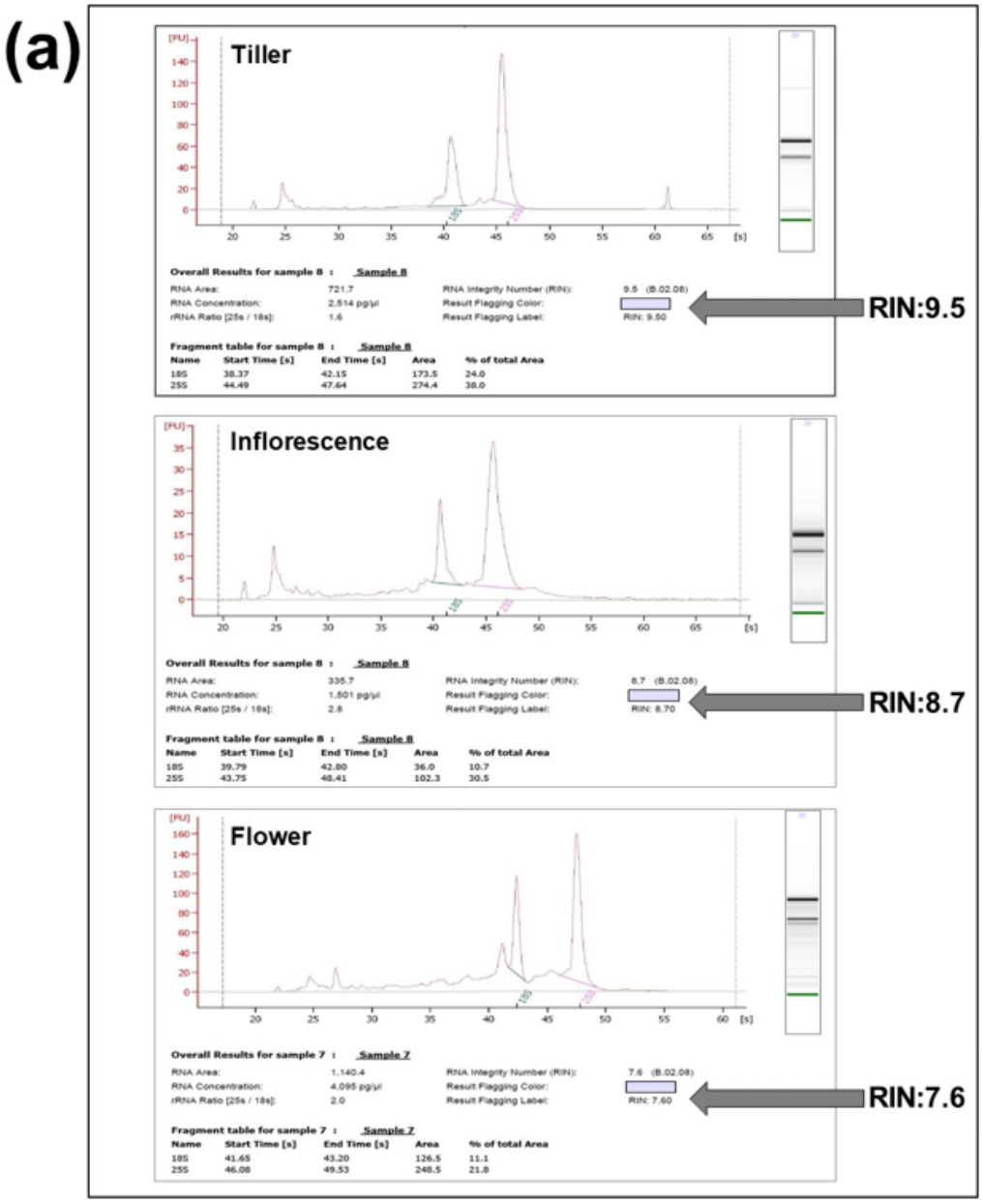

Figure 3. Profile image of intact 25S rRNA, 18S rRNA, and RIN from extracted RNA from (a) Hard tissue samples of tillers, inflorescence, and flowers; (b) soft tissue samples of mature fruit (at ripening index 1), ripe fruit (at ripening index 4), and overripe fruit (at ripening index 7)

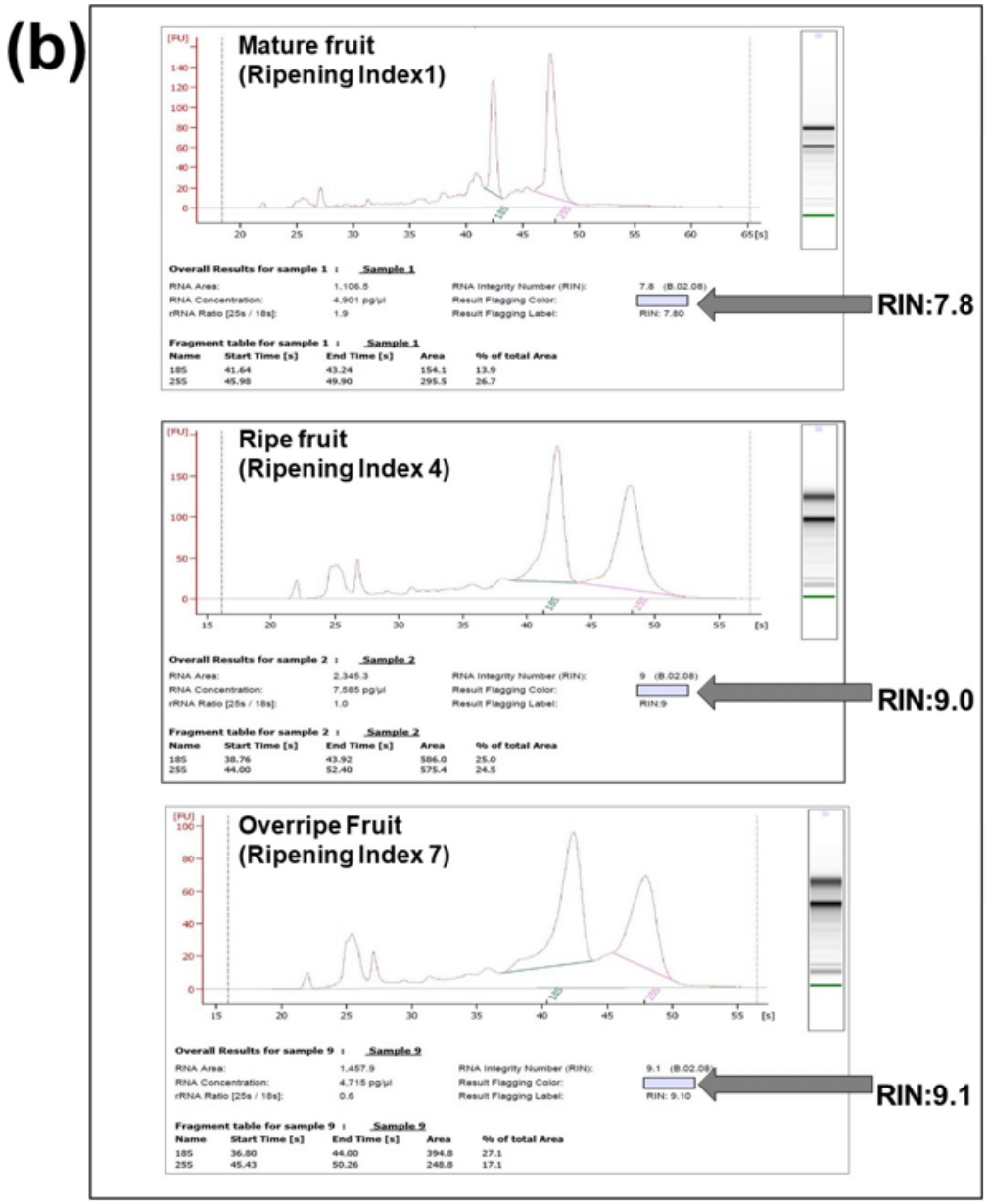

Figure 3. Profile image of intact 25S rRNA, 18S rRNA, and RIN from extracted RNA from (b) soft tissue samples of mature fruit (at ripening index 1), ripe fruit (at ripening index 4), and overripe fruit (at ripening index 7)

DISCUSSION

This study reported protocols for the extraction of total RNA from six pineapple tissues, namely hard tissue samples of the tiller, inflorescence, and flower, as well as soft tissue samples of mature fruit (fruit at ripening index 1), ripe fruit (fruit at ripening index 4), and overripe fruit (fruit at ripening index 7). The KH extraction protocol was first developed to extract RNA from sweet potato (*Ipomoea batatas* (L). Lam.) tissues including the fibrous roots, tuberous roots, flowers, leaves, stems, petioles, thick pigmented roots, and the mature resting tubers (Kim & Hamada, 2005). Hence, it was considered suitable for the extraction of RNA from various pineapple tissues.

The protocol was modified to successfully extract high-quality RNA from both hard and soft tissues of pineapple. β-mercaptoethanol is a strong denaturant commonly used in extraction buffer, with volumes reaching as high as 5% reported (Wang et al., 2010; White et al., 2008). The β-mercaptoethanol concentration in the extraction buffer used in this study was increased from 1% to 2%, thus postulated to increase the rate of RNAse degradation and inhibit its activity, in turn ensuring RNA stability (Azmat et al., 2012; Wang et al., 2010).

Plant tissues contain high levels of polysaccharides, which physiochemically can interact with RNA, thus co-precipitate with RNA, which in turn affects the quality of the RNA. Saturated sodium chloride was used in this study to increase the solubility of polysaccharides, thus reducing the rate of co-precipitation with RNA (Tel-Zur et al., 1999; Wong et al., 2014; X. Li et al., 2011).

The use of CTAB has been reported to be more suitable than SDS for the RNA extraction of samples with leafy-structures including the hard tissues of pineapple (Jordon-Thaden et al., 2015; White et al., 2008; Wong et al., 2014; Yu et al., 2012). As such, CTAB helps to break the thick lignified cell walls of the tissues, separating nucleic acids from polysaccharides (Chaparro-Encinas et al., 2020; Jaakola et al. 2001; Jordon-Thaden et al., 2015). The extraction of high-quality RNA using CTAB from soft pineapple tissues was also done but was found to be unsuitable in this study; hence SDS was used to obtain total RNA. The main factor may be due to the tissue compositions as hard tissues are thickly lignified, whereby the soft pineapple tissues have more water content (85%) and usually are more acidic (pH ~3.5), making the use of SDS more suitable in soft pineapple tissues (Shamsudin et al., 2007). Despite SDS being suitable to extract RNA for soft tissue in this study; however there was no clear correlation between CTAB suitability in hard tissue and SDS in soft tissue as many other protocols have used SDS to extract RNA from hard tissue (Hou et al., 2011; Huded et al., 2018; Ramimoghadam et al., 2012). The incubation at 65°C for extraction was suitable for all hard tissues as it is postulated to increase the efficiency of cell wall degradation.

Phenol was added to the mixture of chloroform and isoamyl alcohol as it provides several advantages in the

process of RNA separation from other contaminants including DNA, proteins, lipids, carbohydrates, and cell debris. Generally, the addition of chloroform alone into a solution that contains RNA forms a biphasic emulsion separating contaminants from total RNA, with the upper layer of the emulsion (the hydrophilic layer) containing the nucleic acids, while the lower layer (the hydrophobic layer) contains other contaminants (Tan & Yiap, 2009). Thus, the use of chloroform alone may not be sufficient to extract RNA without DNA contamination. As such, the acidic conditions developed with the addition of phenol causes the RNA to remain in the upper layer, with most DNA and proteins remaining in the interphase or lower layer (Chomczynski & Sacchi, 2006; Maes & Messens, 1992). Because of this, this study also used acid phenol for separating RNA from contaminants including DNA. On the other hand, the presence of isoamyl alcohol helps to continuously inhibit RNAse activity maintaining RNA stability (Tüzmen et al., 2018). Ethanol is volatile, it is useful in the preparation of samples for NGS purposes, to ensure that the sample is free from chemical residues, as compared to LiCl (Hopkins et al., 2009).

Based on the modification of Kim and Hamada (2005) method, total RNA was successfully extracted from pineapple tissues of different compositions (soft and hard tissues). Total RNA was present in each tissue as seen on Figure 2 with two distinctive band of 25S and 18S representing intact total RNA. Further assays via Nanodrop™ Spectrophotometer (Table 1) and Agilent Bioanalyzer (Figure 3) show total RNA extracted having concentration of more than 50 ng/µl, absorbance ratio (A260:A280) of more than 1.8 and RIN value of more than 7, which are deemed acceptable for RNA sequencing via NGS platform (Kukurba & Montgomery, 2015; Sheng et al., 2017).

CONCLUSION

High-quality RNA was successfully extracted using modified Kim and Hamada (2005) protocol which can be used on tissue with thick lignified cell wall (hard tissues) as well as high water content tissues (soft tissues) of pineapple. The high-quality total RNA reaching minimum concentration, absorbance ratio, and RIN value are applicable for various analytical techniques like reverse transcription polymerase chain reaction (RT-PCR), real-time fluorescent quantitation polymerase chain reaction (qPCR), microarray analysis, and RNA-sequencing via NGS platforms.

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The Effects of Sago (*Metroxylon sagu*) Bark and Frond Waste as Substrates on the Growth and Yield of Grey Oyster Mushrooms (*Pleurotus sajor-caju*)

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ABSTRACT

This study was carried out to examine the effects of sago bark (SB) and sago frond (SF) waste on the growth and yield of grey oyster mushrooms (*Pleurotus sajor-caju*). Nine substrate formulas were studied, including sawdust (SD) alone as a control and the combination of 25:75, 50:50, and 75:25 ratios between SD and SB or SF. The results showed a significant difference in the total colonisation period, total fruiting body yield, and biological efficiency (BE). However, an insignificant difference was determined in the characteristics of the fruiting body for different substrate formulas. The substrates with the ratio of 50SD:50SF and 100 SD are the most suitable substrate formulas for the cultivation of *P. sajor-caju*. One hundred (100) SD achieved the fastest total colonisation period (24.44 days) but there was no significant difference with 75SD:25SF (24.78 days) and also obtained the fastest first harvest (50.33 days). However, 100SD produced a significantly lower total fruiting body yield (77.99 g/bunch) compared to 50SD:50SF, which produced the highest total yield (88.09 g/bunch) and highest BE (17.62%) with a short total colonisation period (26.45 days). The substrates produced high values in cap diameter, stipe length, and effective fruiting bodies.

Keywords: Mushroom cultivation, sago bark, sago frond, waste, yield

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INTRODUCTION

Oyster mushroom (*Pleurotus* sp.) was cultivated worldwide throughout the previous few decades (Royse, 2002). Due to white mycelium production, *Pleurotus* sp. is associated with the white-rot fungi group and under the class basidiomycetes (Tsujiyama & Ueno, 2013). The common name 'oyster' comes from the white shell-like shape of the fruiting body of the mushroom. *Pleurotus* sp. is among the most popular mushroom worldwide in terms of edible basidiomycetes. This species stands in third place in the yield of eatable mushrooms following *Agaricus* and *Lentinula* genus species (Cardoso et al., 2013). *Pleurotus sajor-caju* is the most famous cultured species among these mushrooms and acknowledged for its deliciousness (Zhang et al., 2002).

Mushroom cultivation could be a procedure that helps in waste disposal and environmental waste management. Oyster mushroom cultivation has a major part in managing organic waste wherever dumping becomes difficult (Das & Mukherjee, 2007). An example of organic waste found in Malaysia, particularly in Sarawak, is sago palm tree waste.

Palms come under one of the oldest families of plants on earth (Hisahima, 1995). One of them is sago palm or scientifically known as *Metroxylon sagu*, which is utilised as an essential crop in Southeast Asia due to the high quantity of starch within the trunk. Sago palm is one of the most affordable and convenient food starch sources with the highest starch production per land area compared to different starch products (Rajyalakshmi, 2004). However, problems arise with the expansion of sago starch production in terms of the residues left from sago palm trees, such as trunk bark (cortex) and frond that pollute the environment. To collect edible starch, the whole tree needs to be cut down to collect the inner trunk, and with the increased production of sago palm, a large amount of fibrous waste and trunk bark is left in starch mills, which pollute the environment (Kuroda et al., 2001). According to Ngaini et al. (2014), 15.6 tons of sago woody bark and 7.10 tons of fibrous pith waste from 600 logs of sago palm are generated in a day. The abundance of sago starch waste has created environmental problems as the waste accumulates in fields or is burned (Awg-Adeni et al., 2009).

Therefore, residue utilisation is needed to overcome this issue. One way to utilise sago palm waste is to convert the waste into a substrate for mushroom cultivation. The possibilities for sago palm waste fibre as a mushroom cultivation substrate have not been totally explored. The aim is to determine the alternative substrate for the cultivation of *P. sajor-caju*.

This study was performed to study the performance of various substrate formulas consisting of sago bark (SB), sago frond (SF), and sawdust (SD) waste on the growth and yield of *P. sajor-caju*.

MATERIALS AND METHODS

Pure Culture and Spawn Preparation

Grey oyster mushroom was collected from a local market and identified as *P. sajor-*

caju by using a molecular identification technique. Potato dextrose agar (PDA) medium was used to grow *P. sajor-caju* at 28°C for pure culture and maintained on the PDA medium at 4°C for at most three months. Paddy grains were used for spawn preparation. The spawns were prepared in 850 ml polypropylene plastic bags and PVC tubing was used to close the bags' mouth. The bags were autoclaved at 121°C for 15 min and left to cool at room temperature. Sterilised paddy grains were inoculated with viable mycelia of *P. sajor-caju* from the PDA medium and incubated at 28°C until the mycelia fully colonised the bags.

Substrate Preparation and Spawn Inoculation

The substrates used in this study were mill SD, SB, and SF. The SD was obtained from a local wood factory. All the sago substrates were obtained from a local sago grower in Lundu, Sarawak, Malaysia. Approximately 5 kg of SB and SF were sundried for a week until the waste dried and then ground by a grinding machine into 0.5–1.5 cm length pellets. To determine appropriate substrates and ratios towards grey oyster mushroom cultivation, nine substrate formulas along with SD, SB, and SF alone and the mixtures of 25:75, 50:50, 75:25 ratios consisting of SD with SB and SF (on a dry weight basis) were studied. One hundred per cent (100%) SD substrate was utilised as the control treatment. The data were presented as 100SD, 75SD:25SB, 50SD:50SB, 25SD:75SB, 100SB, 75SD:25SF, 50SD:50SF, 25SD:75SF, and 100SF. After mixing the materials at these ratios, they were prepared using a 100:1:1 ratio for the sample (500 g):rice bran:chalk ($CaCO_3$). Around 65% of the water content was adjusted for the ultimate mixture. Each substrate formula was packed into polyethylene plastic bags and sterilised in an autoclave for 15 min at 121°C and left to cool at room temperature. Next, each bag was inoculated with 10 g spawn per bag and three culture bags were prepared for each substrate formula.

Substrate Incubation and Fruiting Body Harvesting

The inoculated substrate bags were maintained inside the incubation room at 28 °C and 60%–70% relative humidity. The bags were placed on a mushroom shelf outside the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS) once the substrate bags were entirely covered with mycelium. Three mushroom flushes were harvested from every bag for each substrate formula and the total days taken from inoculation until the first harvest was inspected and documented. Any contamination was monitored throughout cultivation. The weight (g/bunch) of individual bunch of mushrooms per bag and proportion of flushes (stipe length and cap diameter) of the harvested fruiting bodies were measured and recorded. The amount of effective fruiting bodies per bag at the first, second, and third flushes and the total means were recorded per bag (g/bag). The collected data were applied to calculate the total yield

and biological efficiency (BE) after the harvesting period end. BE was calculated using the following equation and the results were recorded:

$$\text{BE (\%)} = \frac{Grams\ of\ fresh\ sporophore\ produced}{Grams\ of\ dry\ substrate\ used} \times 100\%$$

Experimental Design and Data Analysis

The research was performed in the Mycology Laboratory, Faculty of Resource Science and Technology, UNIMAS in Malaysia from January 2018 until September 2019. The experiment was arranged in a randomised complete block design with three replications and three cultured bags per treatment in each replication. The collected data were inspected using IBM SPSS Statistics 20. The differences between the substrates were compared using one-way analysis of variance. The test of significance with p-value < 0.05, which is considered significant, was done using Tukey HSD multiple range test. All data were expressed as mean ± standard error.

RESULTS AND DISCUSSION

Effects of Various Substrate Formulas on Grey Oyster Mushroom Morphological Parameters

Nine substrate formulas were tested to determine the growth and yield of *P. sajor-caju*. No visible contamination was observed for all substrate formulations during spawning and cultivation throughout this study. The results in Table 1 display significant differences in the morphological parameters of *P. sajor-caju* cultivated using nine non-identical substrate formulas. The colonisation of *P. sajor-caju* mycelia on the substrate bags was completed between 24.44 and 28.78 days after incubation. The substrate containing 100% SD was completely colonised by mycelia in a shorter time (24.44 days) while 100% SB took the longest time (28.78 days) than other substrate formulas. Mycelium growth in this research was much slower than the previous study (Emiru et al., 2016), showing that oyster mushroom mycelium took 2–3 weeks to colonise the SD substrate bag after complete inoculation. One of the reasons may be due to high carbon/nitrogen (C/N) ratio of SB and SF waste substrate. Naraian et al. (2009) stated that mycelium growth development and pinhead formation rely on the C/N ratio in substrates. A low C/N ratio gives higher results of fruiting bodies and supports better mushroom yield (Royse, 2002). Fresh sago waste contained the C/N ratio of 790.1 (Auldry et al., 2009) while SD contained the C/N ratio of 325.0 (Osunde et al., 2019).

The fastest first harvest collected for *P. sajor-caju* (50.33 days) was recorded from the substrate formula of 100SD and significantly quicker than other substrate formulas, whereas the longest period taken for the first harvest collected (71.86 days) was obtained from the substrate formula of 100SB. The days for the colonisation period of mycelia and the first harvest of

P. sajor-caju were shorter for the substrate formula of 100SD, whereas 100SB took the longest time compared to other substrate formulas. These results supported the finding of a previous study, in which a substrate with a higher ratio of SD has the fastest duration for mycelia to completely colonise the bags (Shah et al., 2004). The current experimentation outcomes agreed with Bugarski et al. (1994), who stated that the first fruiting body occurred on different days, depending on the substrates.

Table 1

Effect of various substrate formulas on morphological parameter and characteristics of fruiting body of Pleurotus sajor-caju

Substrate formula	Total colonization period (day)	First harvest (day)	Cap diameter (mm)	Stipe length (mm)	No. of effective fruiting bodies/ bunch	Fruiting bodies weight (g/bunch)
100SD	24.44 ± 0.84^{a}	50.33 ± 3.35^{a}	7.30 ± 1.10^{a}	6.57 ± 0.71^{a}	2.13 ± 0.42^{a}	26.00 ± 3.75^{ab}
75SD 25SB	25.00 ± 0.89^{ab}	59.46 ± 1.08^{ac}	7.87 ± 0.57^{a}	6.40 ± 0.44^{a}	1.70 ± 0.20^{a}	19.17 ± 2.74^{b}
50SD 50SB	27.22 ± 1.71^{ac}	53.90 ± 4.01^{ac}	7.53 ± 1.11^{ac}	6.87 ± 1.27^{a}	1.70 ± 0.56^{a}	19.99 ± 3.39^{b}
25SD 75SB	27.33 ± 1.34^{ac}	63.10 ± 0.35^{bc}	7.70 ± 0.69^{a}	6.77 ± 0.50^{a}	1.67 ± 0.12^{a}	19.70 ± 0.59^{b}
100SB	28.78 ± 2.14^{c}	71.86 ± 5.17^{b}	8.37 ± 0.55^{b}	7.47 ± 0.81^{a}	1.83 ± 0.50^{a}	21.66 ± 1.75^{ab}
75SD 25SF	24.78 ± 0.19^{a}	67.43 ± 1.63^{bd}	6.70 ± 0.46^{a}	6.13 ± 0.76^{a}	2.16 ± 0.57^{a}	23.18 ± 3.37^{ab}
50SD 50SF	26.45 ± 1.35^{ac}	59.00 ± 7.37^{ad}	8.07 ± 1.00^{a}	6.60 ± 0.40^{a}	2.13 ± 0.70^{a}	29.36 ± 5.19^{a}
25SD 75SF	26.67 ± 0.67^{ac}	69.00 ± 1.30^{bc}	6.87 ± 0.65^{a}	6.07 ± 0.32^{a}	1.93 ± 0.64^{a}	20.39 ± 3.23^{b}
100SF	27.56 ± 0.77^{ac}	67.33 ± 2.03^{bc}	7.300 ± 2.23^{a}	6.67 ± 1.51^{a}	1.77 ± 0.40a	22.55 ± 1.36^{ab}

Note. Significant at 0.05 level in ANOVA; mean values with the same lower-case letters are not significantly different according to Tukey HSD's mean separation test. SD, sawdust; SB, Sago bark; SF, Sago frond

Effects of Various Substrate Formulas on Grey Oyster Mushroom Fruiting Body Characteristics

There were no significant differences in the cap diameter and stipe length of *P. sajor-caju* grown on nine different substrate formulas (Table 1). The highest cap diameter (8.37 cm) was recorded for

the substrate formula 100SB and the lowest diameter (6.70 cm) was documented for the substrate 75SD:25SF. The length of the stipe ranged from 6.07 cm to 7.47 cm (Table 1). One hundred (100) SB recorded a higher mean cap diameter and the longest stipe length than other substrate formulas, although no significant differences were recorded between each substrate formula. By referring to the Federal Agricultural Marketing Authority (FAMA) (2012) on the grade requirement for fresh grey oyster mushroom (MS 2515:2012), all nine substrate formulas produced grade 2 mushrooms. The oyster mushrooms produced in this grade were uniform in size (≤ 20%) and maturity (≤ 10%), fresh (≤ 10%), and clean with reasonably free from deformation (≤ 10%) and damage (≤ 5%). Based on the same standard for the size classification of cap diameter, seven substrate formulas (6.70–7.87 cm) were labelled as size code 2 (M), whereas 100SB (8.37 cm) and 50SD:50SF (8.07 cm) were labelled as size code 3 (L).

According to FAMA (2012), the criteria of effective fruiting body, which is known as the edible mushroom part of mature grey oyster mushroom, can be identified when the cap is a grey, expanded, and has a thin flattened surface, while the gills are white with wider gaps. The mean number of the effective fruiting bodies per bunch was recorded, and the results showed no significant differences between the substrate formulas (Table 1). The results showed that the maximum effective fruiting bodies number of *P. sajor-caju* ranged between 1.70 and 2.16 bodies per bunch for all substrate formulas. Based on a previous study in the same field of interest by Onuoha (2007), SD had the least number of body fruiting production than other substrates, which agreed with the results of this study.

The fruiting bodies weight (g/bunch) of *P. sajor-caju* depends on the cap diameter, stipe length, and total number of effective fruiting bodies per bunch. The fruiting bodies weight is also affiliated with the yield and BE of mushrooms. The weight grown on totally non-identical substrate formulas was significantly different from each other (Table 1). The highest fruiting bodies weight of *P. sajor-caju* (29.36 g/bunch) was obtained from 50SD:50SF substrate. Meanwhile, the lowest fruiting bodies weight was recorded for 75SD:25SB substrate (19.17 g/bunch). The fruiting bodies yield from this study consisting of SD, SB, and SF was lower compared to other studies, as obtained from previous studies. Fasehah and Shah (2017) recorded a mean yield of 42.83 g/bunch using SD as a substrate, whereas Haastrup and Aina (2019) obtained a mean yield of 45.15 g/bunch of mushrooms. However, based on this study alone, SF has a higher potential as a substrate than SB in terms of fruiting bodies yield.

Effects of Various Substrate Formulas on Grey Oyster Mushroom Yield and BE

The main purpose of mushroom cultivators is its yield. For this study, the fruiting bodies of *P. sajor-caju* (in three successive flushes) were gathered from substrate

bags (500 g). *Pleurotus sajor-caju* grown on various substrate formulas showed significant differences in the mushrooms harvested. There was no specific pattern in the mushroom yield (Table 2) from first, second, and third flushes.

Table 2

Effect of various substrate formulas on fruiting bodies *yield and BE of* Pleurotus sajor-caju

Substrate formula	1st flush (g/bag)	2nd flush (g/bag)	3rd flush (g/bag)	Total yield (g/bag)	BE (%)
100SD	25.82 ± 11.01[a]	21.69 ± 4.40[ab]	30.48 ± 4.08[a]	77.99 ± 11.23[a]	15.60 ± 2.24[ab]
75SD 25SB	21.27 ± 4.98[a]	18.01 ± 2.39[a]	18.21 ± 4.16[a]	57.49 ± 8.20[ab]	11.50 ± 1.64[b]
50SD 50SB	21.08 ± 5.40[a]	21.84 ± 4.60[ab]	18.14 ± 3.17[a]	61.06 ± 12.11[a]	12.21 ± 2.42[b]
25SD 75SB	20.07 ± 3.00[a]	16.77 ± 2.13[a]	22.26 ± 3.22[a]	59.10 ± 1.75[ab]	11.82 ± 0.35[b]
100SB	24.19 ± 1.46[a]	19.90 ± 6.59[ab]	20.91 ± 6.61[a]	65.00 ± 5.27[a]	13.00 ± 1.05[ab]
75SD 25SF	21.75 ± 7.38[a]	22.26 ± 4.90[ab]	25.55 ± 9.38[a]	69.56 ± 10.12[a]	13.91 ± 2.02[ab]
50SD 50SF	38.91 ± 10.38[ab]	32.32 ± 4.24[b]	16.86 ± 1.98[a]	88.09 ± 15.57[ac]	17.62 ± 3.11[a]
25SD 75SF	18.21 ± 2.13[ac]	15.89 ± 2.73[a]	27.05 ± 10.70[a]	61.15 ± 9.68[a]	12.23 ± 1.93[b]
100SF	21.06 ± 5.52[a]	23.00 ± 7.08[ab]	23.60 ± 7.40[a]	67.66 ± 4.08[a]	13.53 ± 0.82[ab]

Note. Significant at 0.05 level in ANOVA; mean values with the same lower-case letters are not significantly different according to Tukey HSD's mean separation test. SD, sawdust; SB, Sago bark; SF, Sago frond

Based on Table 2, the total yield of *P. sajor-caju* ranged from 57.49 to 88.09 g/bag. The significantly highest mushroom yield was acquired for 50SD:50SF (88.09 g/bag). A ratio of 75SD:25SB produced the least mushroom harvest (57.49 g/bag), and there was no significant difference from the substrate formula 25SD:75SB (59.10 g/bag).

BE is an excellent parameter to determine the potential of substrate transformation within the fruiting body. Most of the time, substrates with a higher yield produced a higher BE. The higher the BE, the higher the substrate's applicability for mushroom cultivation as the substrate's BE indicates their applicability to support

the expansion of mushroom strain (Megersa et al., 2013). From Table 2, the substrate formula of 75SD:25SB showed the lowest BE (11.50%), whereas 50SD:50SF obtained the highest BE (17.62%). However, there were no significant differences of BE between 75SD:25SB and 25SD:75SB (11.82%), 50SD:50SB (12.21%), and 25SD:75SF (12.23%). In general, the BE of *P. sajor-caju* in the present research is much lower than the study done by Ahmed et al. (2013). These results also differed from the study by Pathmashini et al. (2008), in which the best biological yield for oyster mushroom was achieved for the substrate containing SD. The variations in the yield and BE of *P. sajor-caju* full-grown on completely different substrate formulas are due to variations of the substrate formulas' physical and chemical composition. Based on the fruiting bodies yield and BE from this study, it is shown that SD incorporated with SF enhanced the yield compared to SD alone and incorporated with SB as a substrate.

CONCLUSION

Mushroom production largely depends on the quality of spawn, which is based on substrates. Based on the results, the substrate mixtures of SD with SF have a better fruiting bodies yield and higher BE on *Pleurotus sajor-caju* grown compared to SD with SB and SD alone. The evaluation of SB and SF at different ratios gives positive results, where the ratio with a high amount of SB and SF can produce fruiting body effectively. The present study indicates that SB and SF waste can be used and further developed for local growers' mushroom cultivation. SB and SF can be utilised as one of the substitutional substrates to replace SD in the cultivation of oyster mushroom.

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Dioscorea alata as Alternative Culture Media for Fungal Cultivation and Biomass Production

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ABSTRACT

Dioscorea alata (purple yam) is a tuber crop that contains plenty of nutrients. It is widely cultivated in Thailand, but it is underutilized. In this study, the suitability of purple yam to replace potato dextrose media for fungal growth was investigated. Mushrooms and molds were grown on purple yam dextrose agar (PYDA), whereas yeasts were cultured in purple yam dextrose broth (PYDB). Response surface methodology (RSM) with Box-Behnken design (BBD) was used to optimize the culture conditions for *Saccharomyces cerevisiae* biomass production. The growth profile of *S. cerevisiae* in PYDB under optimized culture conditions was also studied. All test mushrooms and molds recorded the highest colony diameter and mycelial dry weight on PYDA containing 40% purple yam. Similar to mushrooms and molds, yeasts in PYDB with 40% purple yam showed the highest number of cells. The growth of fungi on purple yam dextrose media was significantly higher than those on potato dextrose media under standard conditions. The optimal conditions from the RSM results for the biomass production of *S. cerevisiae* in PYDB were purple yam concentration of 49.61%, dextrose concentration of 4.87%, pH value of 5.74, and inoculum size of 7.00%. The biomass of *S. cerevisiae* in PYDB under the optimal conditions obtained from the results of the optimization by RSM was thirty times higher than *S. cerevisiae* biomass in potato dextrose broth under standard conditions. Our results suggest that purple yam could be an alternative to potato dextrose media for fungal cultivation.

Keywords: Biomass production, Box-Behnken design, *Dioscorea alata*, fungal growth, purple yam, response surface methodology, statistical optimization

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INTRODUCTION

Fungi are eukaryotic organisms comprising multicellular (molds and mushrooms) and unicellular (yeasts) fungi, and are chemoheterotrophs which assimilate the organic compounds as energy and nutritional sources for their growth (Singh & Kapoor, 2010). Macro and micronutrients are essential for fungal growth and reproduction (Wongjiratthiti & Yottakot, 2017).

Potato dextrose media are major media for the isolation and cultivation of fungi under laboratory conditions (Laurie et al., 2015). In Thailand, potato is rarely cultivated and only grown in three provinces (Tak, Chiang Mai, and Chiang Rai provinces) (Kittipadakul et al., 2016), and is so much expensive than the other local crops (https://www.simummuangmarket.com/en). The fungal cultivation using powdered potato dextrose media (commercial) is expensive. The utilization of agricultural crops as alternative culture media in the cultivation of fungi was studied (Adesemoye & Adedire, 2005; Amadi & Moneke, 2012; Nguyen & Ranamukhaarachchi, 2020; Wongjiratthiti & Yottakot, 2017). There are underutilized agricultural crops in Thailand. *Dioscorea alata* (purple yam) is one of the underutilized local crops, although it contains many essential nutrients such as carbohydrate, protein, potassium, sodium, phosphorus, calcium, and magnesium (Chandrasekara & Kumar, 2016; Wanasundera & Ravindran, 1994), and is widely cultivated in the north-eastern region of Thailand especially Sakon Nakhon and Kalasin provinces. The utilization of purple yam tuber as alternative culture media for fungal cultivation is seldom reported. Moddaeng and Khompun (2019) showed that purple yam was a suitable alternative tuber to media preparation for the cultivation of *Curvularia lunata* and *Fusarium solani*.

Saccharomyces cerevisiae is useful yeast involved in many industrial processes such as starter cultures, enzyme production (Arevalo-Villena et al., 2017), and alcoholic fermentation (Shiroma et al., 2013). The growth of yeast depends on different culture conditions, such as culture medium components and their concentrations, pH of culture medium, inoculum size, and others (Jiru et al., 2017; Mad Saad et al., 2016; Shu et al., 2020). The high biomass yield can be beneficial for industries related to *S. cerevisiae*. The optimization of cultivation conditions using single factor experiment in a step-by-step fashion cannot understand an interaction between the factors. Response surface methodology (RSM) is successfully operated to optimize the process factors and it can give data about the interaction between factors (Dinarvand et al., 2017; Sharmila et al., 2013). Box-Behnken design (BBD) is an experimental design for RSM, and it can help to reduce the number of experimental trials (Tayeb et al., 2018).

The possibility of purple yam utilization as alternative culture media for fungal cultivation was studied by assessing the effect of purple yam at various concentrations on the mycelial growth of test mushrooms

and molds on purple yam agar media and the growth of test yeasts in purple yam broth media. The culture conditions for *S. cerevisiae* biomass production in purple yam broth medium such as medium (purple yam and dextrose concentration), pH of the culture medium, and inoculum size were optimized through RSM based on BBD. The growth profile of *S. cerevisiae* in purple yam broth medium under the optimized culture conditions was also investigated. The biomass production of *S. cerevisiae* in purple yam broth medium was studied as an example of the utilization of purple yam in further fungal cultivation and biomass production.

MATERIALS AND METHODS

Test Fungi and Crop Materials

Three species of each mushroom, mold, and yeast were used as representatives of fungi in the study of purple yam utilization for fungal cultivation. The test mushrooms were *Pleurotus eryngii* (PE), *Pleurotus sajor-caju* (*Fr.*) *Singer* (PS), and *Pleurotus ostreatus* (Fr.) Kummer (PO). The test molds were *Bipolaris oryzae* DOAC 1760, *Fusarium oxysporum*, and *Penicillium* sp. The test yeasts were *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, and *Rhodotorula* sp. All test organisms were collected in the Program of Biology, Faculty of Science and Technology, Sakon Nakhon Rajabhat University, Thailand. Purple yam (*Dioscorea alata*) was obtained from the local farmers in Sakon Nakhon province, Thailand.

Media Preparation

The growth of test fungi was carried out in potato dextrose media as control media and purple yam dextrose media as test media. Different media were formulated, namely purple yam dextrose media [purple yam dextrose agar (PYDA), purple yam dextrose broth (PYDB)], potato dextrose media [potato dextrose agar (PDA), potato dextrose broth (PDB)] (Difco™, France) and dextrose media [dextrose agar (DA), dextrose broth (DB)]. The purple yam infusion was prepared according to the method of Wongjiratthiti and Yottakot (2017). The purple yam was washed with water, peeled, and diced. The diced purple yam at various concentrations was boiled in distilled water at 100°C for 15 min and then filtered through cheesecloth. The filtrates were added with different dextrose concentrations (HiMedia Laboratories, India). The DB contains only 2.0% (w/v) of dextrose. The agar media (PYDA and DA) were prepared by adding 2.0% (w/v) of agar (Biomark Laboratories, India) to broth media. The pH of all media was adjusted with HCl or NaOH before sterilization in an autoclave at 121°C for 15 min. After sterilization, PDA, PYDA, and DA were allowed to cool down to around 60°C and poured into petri dishes (9 cm diameter). The PDB, PDA, DA, and DB were used as control media. The pH of all media was adjusted to pH 5.1 ± 0.2.

Effect of Purple Yam at Different Concentrations on the Mycelial Growth of Mushrooms and Molds

Pure cultures of the test mushrooms and molds from PDA slants at 4°C were transferred on PDA plates. All plates were incubated at 25°C for 168 h. An agar plug (6 mm diameter) of each mushroom and mold mycelium was placed onto the center of the test agar media under aseptic condition and incubated at 25°C for 168 h. The mycelial growth of test filamentous fungi was evaluated on six different agar media including PYDA at four concentrations of purple yam [10, 20, 30, and 40% (w/v)], DA, and PDA. After incubation, the colony diameter was measured using a vernier caliper, and mycelial dry weight was determined. To determine the mycelial dry weight, the mycelium with agar media was melted in a microwave oven for 20 s at 800 W. After that, the mycelium was filtered from the medium, rinsed with hot distilled water (about 60°C), and dried in a hot air oven at 105°C until constant in weight (Vargas-Isla & Ishikawa, 2008). The experiment was performed in triplicates. The results were analyzed by one-way analysis of variance (ANOVA) and mean differences were considered significant at $p < 0.05$ by Tukey's HSD post hoc multiple comparison test.

Effect of Purple Yam at Different Concentrations on the Growth of Yeasts

Pure cultures of the test yeasts from PDA slants at 4°C were transferred into flasks containing PDB. Flasks were incubated at 30°C under shaking at 180 rpm for 24 h. After 24 h, the cells were harvested by centrifugation at 5,635 x g for 10 min and were washed twice with sterile 0.85% (w/v) NaCl. The yeast precipitates were resuspended in sterile distilled water. The inoculum density of the yeast suspensions was adjusted to an optical density at 600 nm (OD600) of 0.34 (McFarland standard No. 1, India). Each flask of the broth media was inoculated with 3% (w/v) of inoculum and incubated in a shaker at 30°C and 180 rpm for 24 h. The growth of test yeasts in six broth media including PYDB at four concentrations of purple yam [10, 20, 30, and 40% (w/v)], DB, and PDB was enumerated using a hemocytometer (Wongjiratthiti & Yottakot, 2017). The experiment was done in triplicates. The results were analyzed by one-way ANOVA and mean differences were considered significant at $p < 0.05$ by the Tukey method.

Optimization of Culture Conditions for *Saccharomyces cerevisiae* Biomass Production in PYDB

Purple yam concentration, dextrose concentration, pH, and inoculum size were optimized by response surface methodology (RSM) using Box-Behnken design for the biomass production of *Saccharomyces cerevisiae* in PYDB. The conditions for the biomass production in *S. cerevisiae* were optimized as described in Table 1. The number of *S. cerevisiae* was counted with a hemocytometer under a microscope after 24 h of incubation.

Table 1
Experimental code and levels of the factors in BBD

Factors	Code	Levels		
		-1	0	1
Purple yam concentration (% w/v)	*A*	10	30	50
Dextrose concentration (% w/v)	*B*	1	3	5
pH	*C*	3	5	7
Inoculum size (% w/v)	*D*	3	5	7

Determination of Growth Profile of *Saccharomyces cerevisiae* in PYDB

Saccharomyces cerevisiae cultured in 100 mL PYDB medium with optimal conditions from RSM was incubated at 30°C under shaking condition of 180 rpm. The growth of *S. cerevisiae* was taken every 6 h during an incubation period of 48 h.

RESULTS AND DISCUSSION

Effect of Purple Yam at Different Concentrations on the Mycelial Growth of Mushrooms and Molds

Results of the experiment clearly indicated that significantly highest colony diameter and mycelial dry weight of all test mushrooms and molds were found on PYDA media at 40% (w/v) purple yam (Figures 1A-C and 2A-C). The colony diameter and mycelial dry weight of all mushrooms and molds on PYDA at 40% (w/v) purple yam were significantly higher than PDA and DA (control media) suggesting that purple yam is rich in essential nutrients for fungal mycelial growth. DA cannot promote the growth of all mushrooms and molds mycelium. Earlier findings by Moddaeng and Khompun (2019) also found that purple yam dextrose agar was a suitable medium for the cultivation of *Curvularia lunata* and *Fusarium solani*. Fungal growth under laboratory conditions is dependent on the nutrients in media, which are important for growth, survival, and reproduction (Hoa & Wang, 2015; Wongjiratthiti & Yottakot, 2017). Chandrasekara and Kumar (2016) reported that purple yam has a higher carbohydrate, energy, vitamins (thiamine, riboflavin, and vitamin B6), and minerals (calcium, magnesium, and potassium) than potato. These nutrients are important factors for the growth of molds (Uraz & Özer, 2014) and mushrooms (Bellettini et al., 2019).

Although, the mycelial growth of PE and PS at 20% (w/v) purple yam concentration was less than PDA, the increase of the purple yam concentration up to 40% (w/v) led to the mycelial growth of PE and PS was higher than PDA. The concentration of culture media components had an effect on mycelial growth (Xiao et al., 2004). The result indicated that PYDA media at 40% (w/v) purple yam had the possibility to use as substitutes for PDA in the cultivation of mushrooms and molds mycelium.

Effect of Purple Yam at Different Concentrations on the Growth of Yeasts

The effect of purple yam at different concentrations of growth media on *S.*

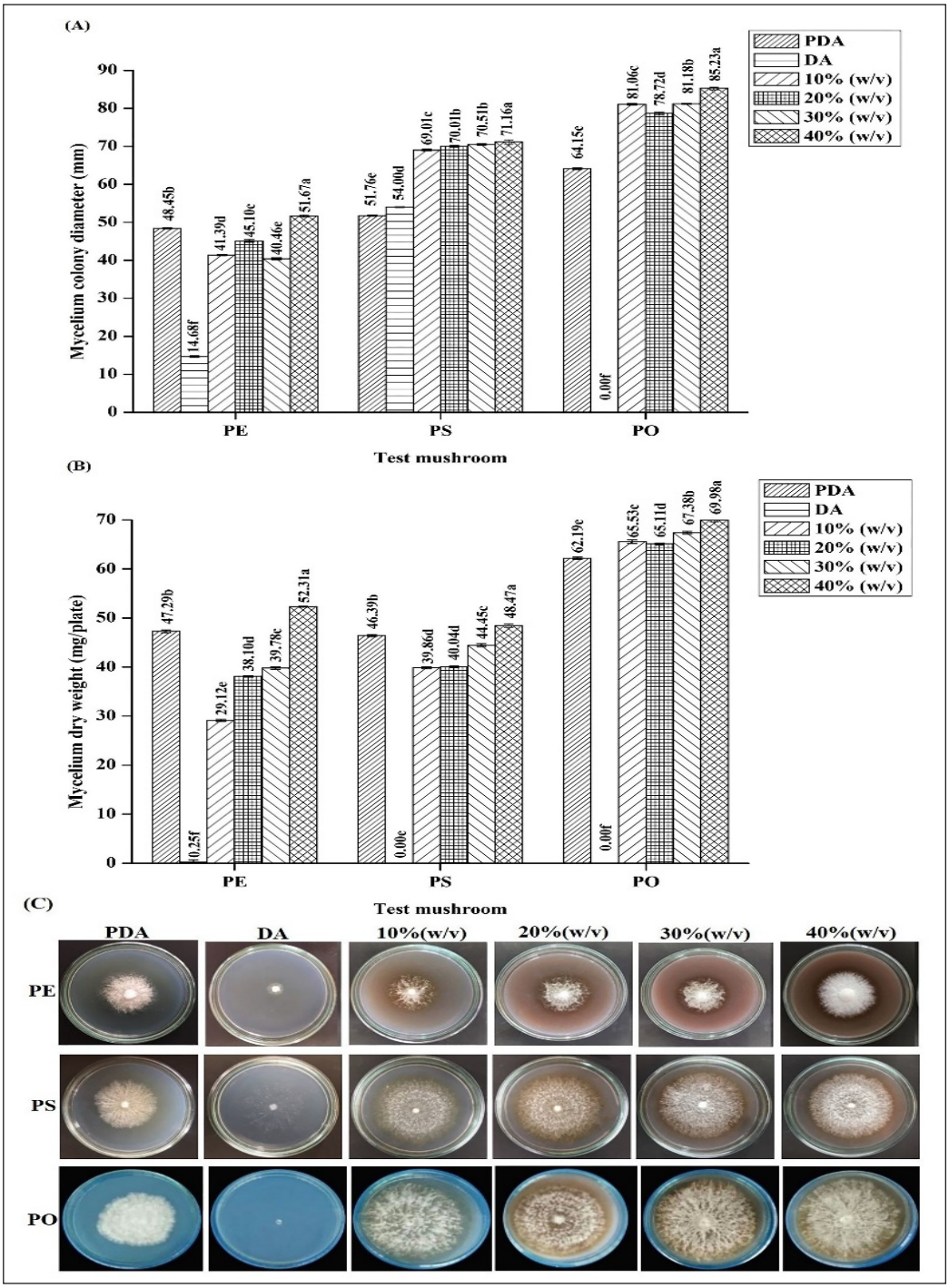

Figure 1. (A) Colony diameter, (B) dry weight, and (C) mycelium growth of *Pleurotus eryngii* (PE), *Pleurotus sajor-caju* (Fr.) Singer (PS), and *Pleurotus ostreatus* (Fr.) Kummer (PO) on different agar media after 168 h of incubation at 25 °C

Note. A pair of averages with different letters is considered significantly different at $p < 0.05$

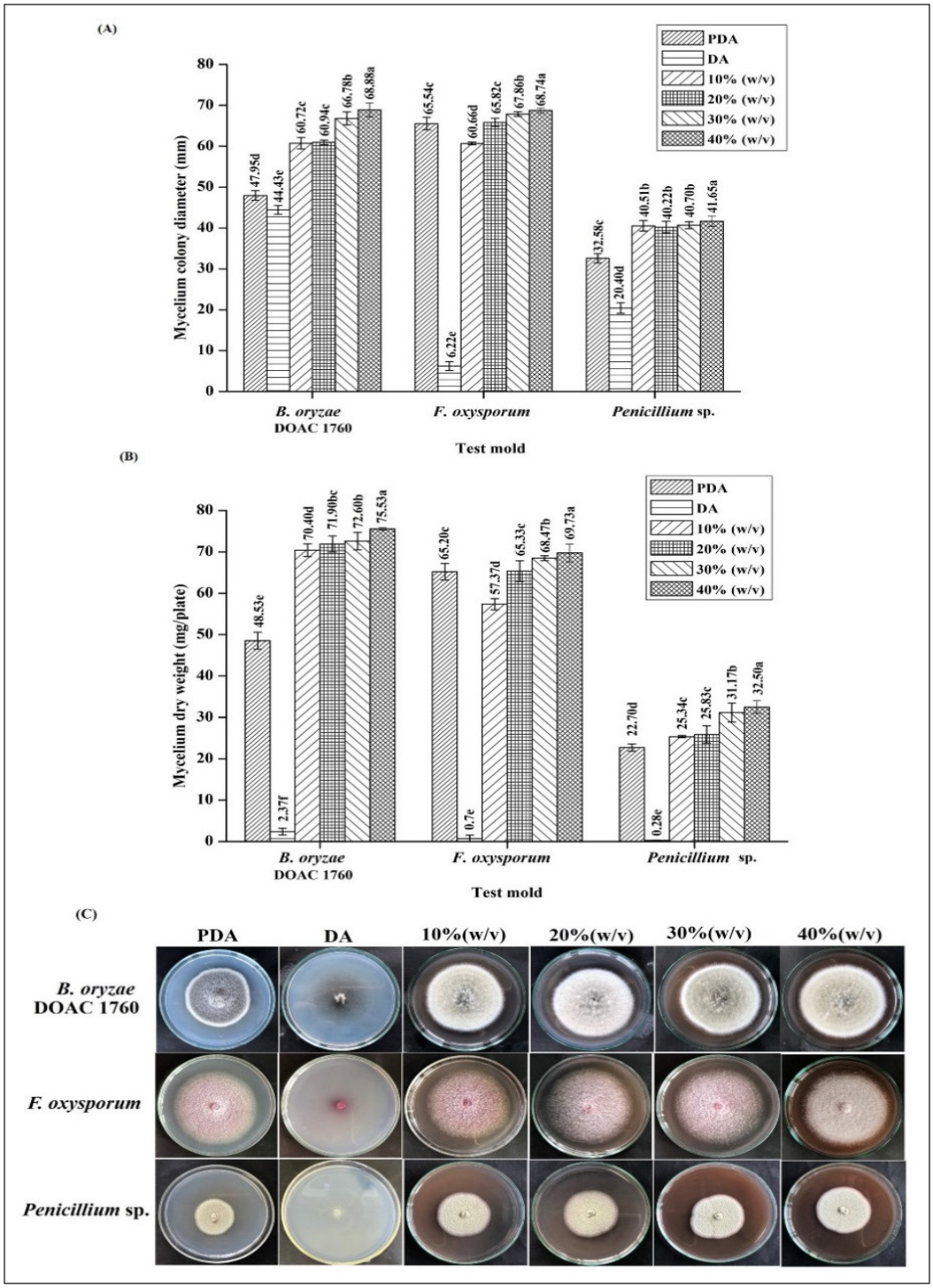

Figure 2. (A) Colony diameter, (B) dry weight, and (C) mycelium growth of *Bipolaris oryzae* DOAC 1760, *Fusarium oxysporum*, and *Penicillium* sp. on different agar media after 168 h of incubation at 25°C
Note. A pair of averages with different letters is considered significantly different at $p < 0.05$

cerevisiae, *S. boulardii*, and *Rhodotorula* sp. was tested (Figure 3). The highest number of *S. cerevisiae*, *S. boulardii*, and *Rhodotorula* sp. were obtained in PYDB media at 40% (w/v) purple yam with the cell number of $7.69 \times 10^6 \pm 0.40$, $3.09 \times 10^7 \pm 0.20$, and $3.45 \times 10^7 \pm 0.25$ cells/mL, respectively. The growth of all test yeasts in 40% (w/v) PYDB was significantly higher than in the standard culture medium (PDB), DB, and the other purple yam concentrations, because purple yam is rich in sugars, carbohydrates, proteins, vitamins (riboflavin, vitamin B6, and thiamine), and minerals (calcium, magnesium, phosphorus, sodium, and potassium) (Chandrasekara & Kumar, 2016; Wanasundera & Ravindran, 1994). These nutrients play an important role for cellular metabolism (such as the biosynthesis of nucleic acids, phospholipids, and ATP), growth and multiplication of two test *Saccharomyces* species (Farinazzo et al., 2017; Walker & Stewart, 2016) and *Rhodotorula* sp. (especially phosphorus) (Kot et al., 2019). Thiamine, which is high in purple yam (Chandrasekara & Kumar, 2016), is an essential coenzyme in carbohydrate and amino acid metabolism, and could promote the growth of yeasts (Hucker et al., 2016; Li et al., 2010).

Optimization of Culture Conditions for *Saccharomyces cerevisiae* Biomass Production in PYDB

Saccharomyces cerevisiae is recognized as a model organism (Karathia et al., 2011; Parapouli et al., 2020) and a most valuable species in various industrial applications (Arevalo-Villena et al., 2017; Shiroma et al., 2013; Parapouli et al., 2020). The optimization of culture conditions for *S. cerevisiae* biomass production in PYDB

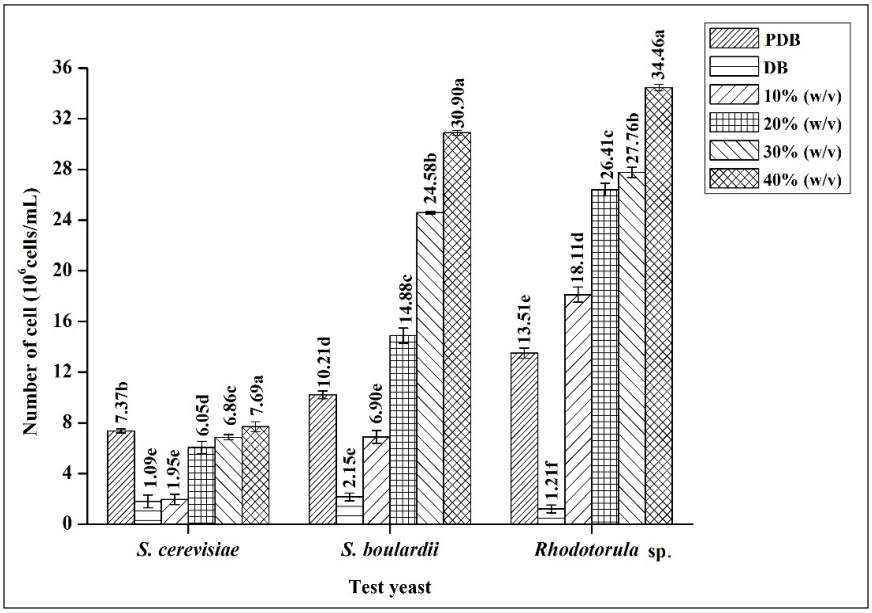

Figure 3. The growth of the test yeasts in different broth media for 24 h
Note. A pair of averages with different letters is considered significantly different at $p < 0.05$

was examined through RSM with BBD. Twenty-nine experiments based on BBD with three levels of each factor and their actual and predicted responses are shown in Table 2. The experimental results received from BBD (actual values) were fitted to a second-order polynomial equation to predict the optimal culture conditions of *S. cerevisiae* in PYDB. The number of *S. cerevisiae* in PYDB follows the response surface regression model [1].

Table 2
The Box–Behnken experimental design for Saccharomyces cerevisiae *biomass production and their actual and predicted responses*

STD	Factor *A*	Factor *B*	Factor *C*	Factor *D*	Biomass (cells/mL)	
					Actual value	Predicted value
1	10	1	5	5	5.60×10^7	5.94×10^7
2	50	1	5	5	1.42×10^8	1.49×10^8
3	10	5	5	5	8.64×10^7	8.60×10^7
4	50	5	5	5	2.04×10^8	2.08×10^8
5	30	3	3	3	2.02×10^7	4.51×10^7
6	30	3	7	3	4.46×10^7	5.35×10^7
7	30	3	3	7	2.12×10^7	1.88×10^7
8	30	3	7	7	1.45×10^8	1.27×10^8
9	10	3	5	3	6.72×10^7	6.57×10^7
10	50	3	5	3	1.83×10^8	1.73×10^8
11	10	3	5	7	7.62×10^7	9.06×10^7
12	50	3	5	7	1.90×10^8	1.96×10^8
13	30	1	3	5	1.85×10^7	1.65×10^7
14	30	5	3	5	2.90×10^7	3.65×10^7
15	30	1	7	5	5.59×10^7	5.23×10^7
16	30	5	7	5	1.11×10^8	1.18×10^8
17	10	3	3	5	6.70×10^4	-1.60×10^7
18	50	3	3	5	6.09×10^7	4.97×10^7
19	10	3	7	5	1.03×10^6	2.10×10^6
20	50	3	7	5	1.41×10^8	1.48×10^8
21	30	1	5	3	1.03×10^8	9.55×10^7
22	30	5	5	3	1.75×10^8	1.62×10^8
23	30	1	5	7	1.40×10^8	1.44×10^8
24	30	5	5	7	1.64×10^8	1.62×10^8
25	30	3	5	5	1.49×10^8	1.51×10^8
26	30	3	5	5	1.59×10^8	1.51×10^8
27	30	3	5	5	1.49×10^8	1.51×10^8
28	30	3	5	5	1.38×10^8	1.51×10^8
29	30	3	5	5	1.59×10^8	1.51×10^8

Note. STD = Standard; Factor *A* = Purple yam concentration; Factor *B* = Dextrose concentration; Factor *C* = pH; Factor *D* = Inoculum size

$$Y = -438662875 + (2240225A) + (17592250B) + (178699166.66667C) + (-9565833.3333334D) + (199787.4999999AB) + (504125AC) + (-135\,62.5AD) + (2838437.5BC) + (-3031875BD) + (6258437.5CD) + (-4401\,3.3333333334A^2) + (-1993677.0833334B^2) + (21902458.333333C^2) + (-627614.58333333D^2) \quad [1]$$

where Y is the predicted biomass of *S. cerevisiae* (cells/mL), and A, B, C, and D are the coded variables of purple yam concentration, dextrose concentration, pH, and inoculum size, respectively.

ANOVA for the investigation of the model adequacy is presented in Table 3. The result of ANOVA with p-value of 5.45×10^{-9} and F-value of 42.14 revealed that the model was highly significant. The coefficient of determination R^2 of 0.9768

Table 3
ANOVA for response surface regression model of Saccharomyces cerevisiae *biomass production*

Source	SS	df	MS	F-value	p-value
Model	1.08×10^{17}	14	7.72×10^{15}	42.14	5.45×10^{-9}*
A	3.37×10^{16}	1	3.37×10^{16}	184	1.90×10^{-9}*
B	5.45×10^{15}	1	5.45×10^{15}	29.73	8.51×10^{-5}*
C	1.02×10^{16}	1	1.02×10^{16}	55.85	2.99×10^{-6}*
D	1.69×10^{15}	1	1.69×10^{15}	9.26	0.0087*
AB	2.55×10^{14}	1	2.55×10^{14}	1.39	0.2574
AC	1.62×10^{15}	1	1.62×10^{15}	8.87	0.0099*
AD	1.17×10^{11}	1	1.17×10^{11}	0.006	0.9372
BC	5.15×10^{14}	1	5.15×10^{14}	2.81	0.1156
BD	5.88×10^{14}	1	5.88×10^{14}	3.20	0.0948
CD	2.50×10^{15}	1	2.50×10^{15}	13.67	0.0023*
A^2	2.01×10^{15}	1	2.01×10^{15}	10.96	0.0051*
B^2	4.12×10^{14}	1	4.12×10^{14}	271.57	0.1558
C^2	4.97×10^{14}	1	4.97×10^{14}	202.49	1.45×10^{-10}*
D^2	4.08×10^{12}	1	4.08×10^{12}	0.22	0.6440
Lack of fit	2.27×10^{15}	10	2.27×10^{14}	3.07	0.1455
Pure error	2.95×10^{14}	4	7.39×10^{12}		
Cor total	1.10×10^{17}	28			
$R^2 = 0.9768$ Adjusted $R^2 = 0.9536$ Predicted $R^2 = 0.8777$					

Note. * Significant ($p < 0.05$); SS = Sum of squares; df = Degrees of freedom; MS = Mean square; A = Purple yam concentration; B = Dextrose concentration; C = pH; D = Inoculum size; AB = Purple yam concentration × Dextrose concentration; AC = Purple yam concentration × pH; AD = Purple yam concentration × Inoculum size; BC = Dextrose concentration × pH; BD = Dextrose concentration × Inoculum size; CD = pH × Inoculum size; A^2 = Purple yam concentration × Purple yam concentration; B^2 = Dextrose concentration × Dextrose concentration; C^2 = pH × pH; D^2 = Inoculum size × Inoculum size

and the adjusted coefficient of determination R^2 of 0.9536 were high and close to 1, indicating the good correlation between the factors and the response (Kamal et al., 2019) and the suitability of the model (Hu et al., 2016). Besides, the lack of fit was not significant, which displayed that the regression equation of this study is good and acceptable (Islam Shishir et al., 2016). These results, therefore, indicated that the response surface regression model is reliable and accurate in predicting and investigating the optimal conditions for the biomass production of *S. cerevisiae* in PYDB.

As shown in Table 3, all independent variables in this optimization consist of purple yam concentration, dextrose concentration, pH, and inoculum size greatly affected the biomass production of *S. cerevisiae* in PYDB, because the *p*-value of the linear term of every variable (*A*, *B*, *C*, and *D*) and quadratic term of purple yam concentration (A^2) and pH (C^2) were very significant. The interaction effect of purple yam concentration and pH (*AC*), as well as pH and inoculum size (*CD*) also affected *S. cerevisiae* growth in PYDB.

The three-dimensional (3D) response surface curves and their corresponding two-dimensional (2D) contour plots are generally the graphical representations of the relationship between the variables and response, which were generated from the regression model (Figures 4A-F). Each 3D response surface with 2D plot demonstrates the predicted response of the combinations of two test variables with the other two variables were maintained at their center value. The impact of independent variables, the impact of the interaction effect of variables, and the optimal value of each variable for maximum biomass of *S. cerevisiae* in PYDB were also discovered with these graphical plots.

The concentration of components in the culture medium is one of the important factors that should be optimized for the highest biomass to accomplish the highest advantage of the production (Reihani & Khosravi-Darani, 2019). There are only two components in the PYDB culture medium, purple yam, and dextrose. An increase in the concentration of purple yam (Figures 4A, 4B, and 4C) and dextrose (Figures 4A, 4D, and 4E) resulted in increased biomass production. The predicted optimal concentration of purple yam and dextrose in PYDB were 49.61 and 4.87%, respectively. Hezarjaribi et al. (2016) found that the increase of glucose up to 5% also gave the highest biomass and single cell protein production from *S. cerevisiae*. Purple yam and dextrose are carbon and energy sources (Chandrasekara & Kumar, 2016; Wanasundera & Ravindran, 1994), both of which are essential sources for *S. cerevisiae* growth (Mitterdorfer et al., 2001). Dextrose is known to affect numerous processes in *S. cerevisiae*, but in fact, dextrose also has a profound effect on yeast gene transcription (Newcomb et al., 2003).

The pH in the culture medium is an important factor that affects the growth of *S. cerevisiae* (Kasemets et al, 2007). The predicted optimal pH of *S. cerevisiae* in PYDB was 5.74, resulting in the maximum

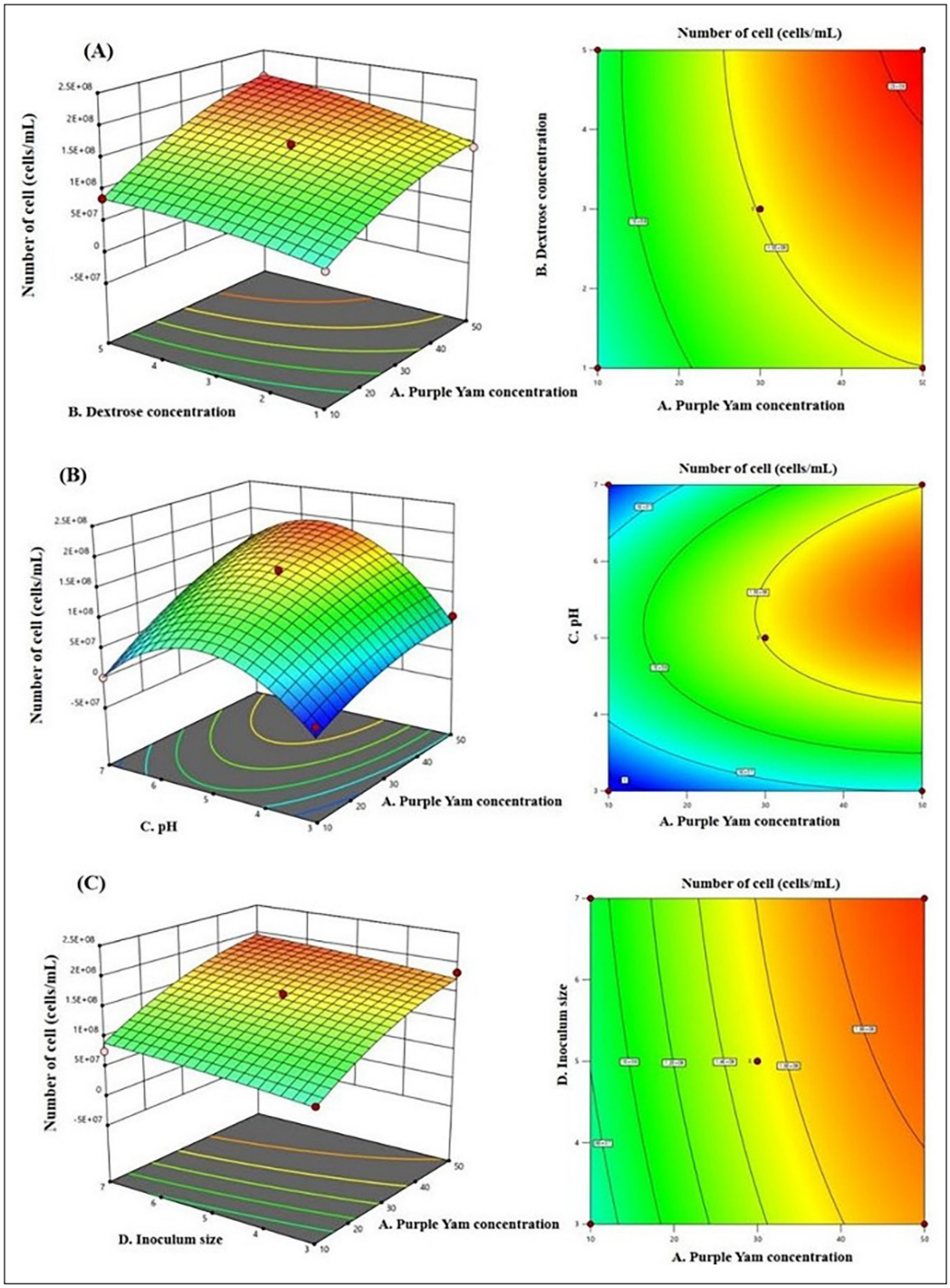

Figure 4. 3D response surface plots (left) and 2D contour plots (right) show the effect of (A) purple yam concentration and dextrose concentration; (B) purple yam concentration and pH; (C) purple yam concentration and inoculum size on the number of *Saccharomyces cerevisiae*

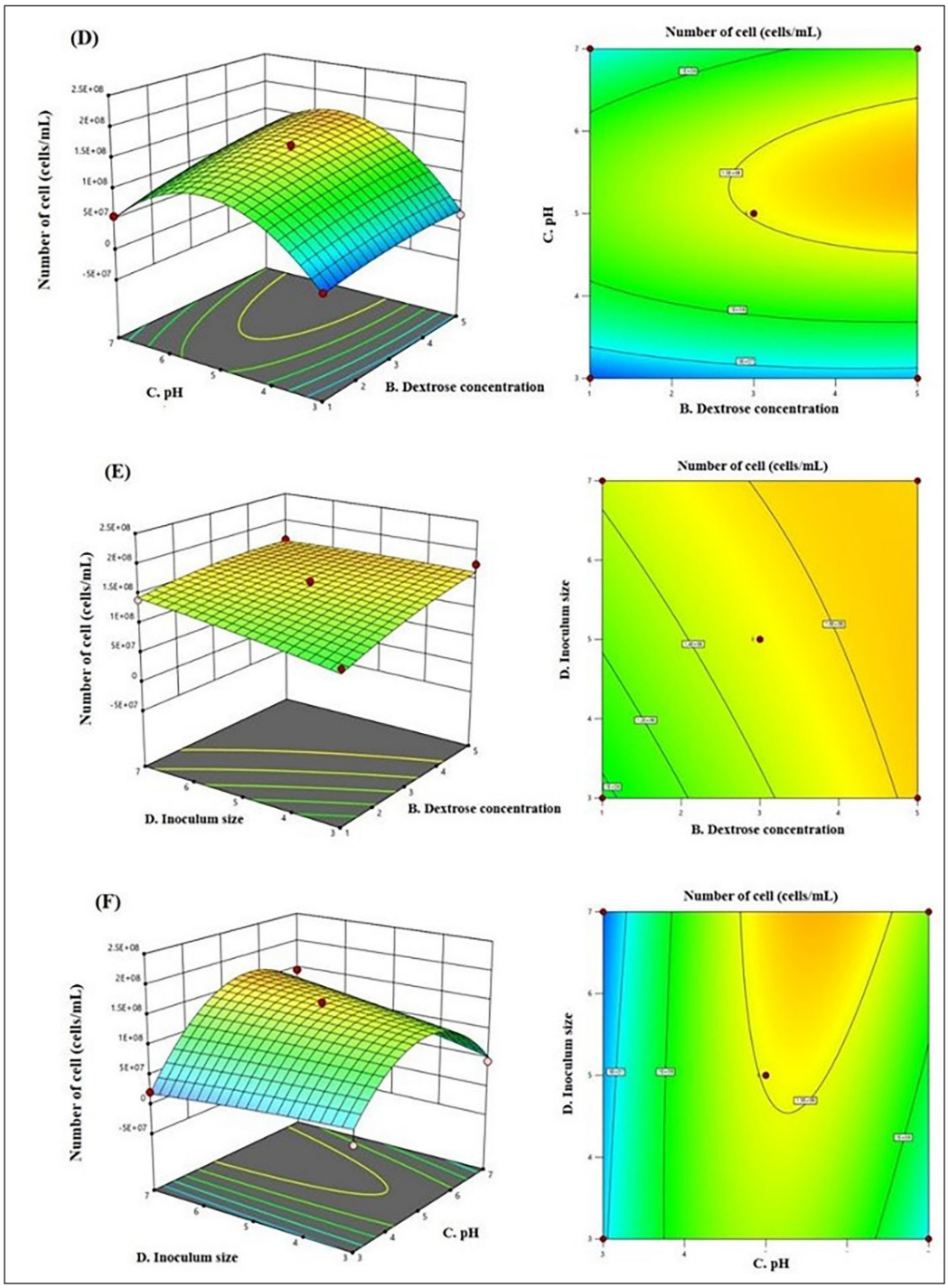

Figure 4 (continue). 3D response surface plots (left) and 2D contour plots (right) show the effect of (D) dextrose concentration and pH; (E) dextrose concentration and inoculum size; (F) pH and inoculum size on the number of *Saccharomyces cerevisiae*

biomass production of *S. cerevisiae* in PYDB (Figures 4B, 4D, and 4F). Most yeast thrives under acidic conditions than at neutral or alkaline conditions (Canadell et al., 2015; Narendranath & Power, 2005). However, the biomass production of *S. cerevisiae* decreased under extremely acidic conditions (Noé Arroyo-López et al., 2009). The optimal pH of *S. cerevisiae* growth in each culture medium is different. The optimal pH for biomass production of *S. cerevisiae* in mineral salts medium supplemented with date extract was 5.35 (Kara Ali et al., 2017), while a pH value of 4.6 is optimum for *S. cerevisiae* in sweet potato dextrose broth (Wongjiratthiti & Yottakot, 2017).

The inoculum size not only has a direct impact on the overall production yield (Ginovart et al., 2011; Jiru et al., 2017; Kamal et al., 2019; Reihani & Khosravi-Darani, 2019), but it also affects the production cost (Reihani & Khosravi-Darani, 2019). The increase of the inoculum size has a positive effect on the production of *S. cerevisiae* biomass in PYDB (Figures 4C, 4E, and 4F). The shortest lag phase and time for the first cell division were attained with the largest inoculum size (Ginovart et al., 2011). The optimal inoculum size differs for each type of microorganism in each type of culture medium (Reihani & Khosravi-Darani, 2019). The inoculum size of 7.00% was an optimal condition for the biomass production of *S. cerevisiae* in PYDB.

The interaction effect between purple yam concentration and pH is observed in Table 3 and Figure 4B, as well as between pH and inoculum size (Table 3 and Figure 4F). The ellipse was found to be slightly oblique in the contour plot of Figures 4B and 4F. The optimal pH of *S. cerevisiae* in PYDB was slightly more neutral when the purple yam concentration was higher (Figure 4B), likewise, when the inoculum size was larger (Figure 4F). The yeast utilizes nutrients in the culture medium, and produces organic acids as the metabolic product, which affects to increase the acidity of the culture medium (Porro & Branduardi, 2017).

The purple yam concentration of 49.61% and the dextrose concentration of 4.87% at pH value of 5.74 with 7.00% inoculum size were the predicted optimal culture conditions for the biomass production of *S. cerevisiae* in PYDB medium. The predicted *S. cerevisiae* biomass under these optimized conditions was 2.24×10^8 cells/mL (Figure 5).

The validation of the predicted statistical result at optimized conditions was performed, and the comparison of this result with the predicted number of cells in PYDB under optimized conditions from the response regression model and the number of cells in PDB under standard conditions is shown in Figure 6. The experimental value of 2.20×10^8 cells/mL resembles with the predicted value (2.24×10^8 cells/mL). A total of 2.20×10^8 cells/mL was recorded when growing on PYDB under the optimized culture conditions, which is thirty times higher than the number of *S. cerevisiae* in PDB under standard conditions of 7.37×10^6 cells/mL. Therefore, in addition to the acceptable model, PYDB could be satisfactorily used for the biomass production of *S. cerevisiae*.

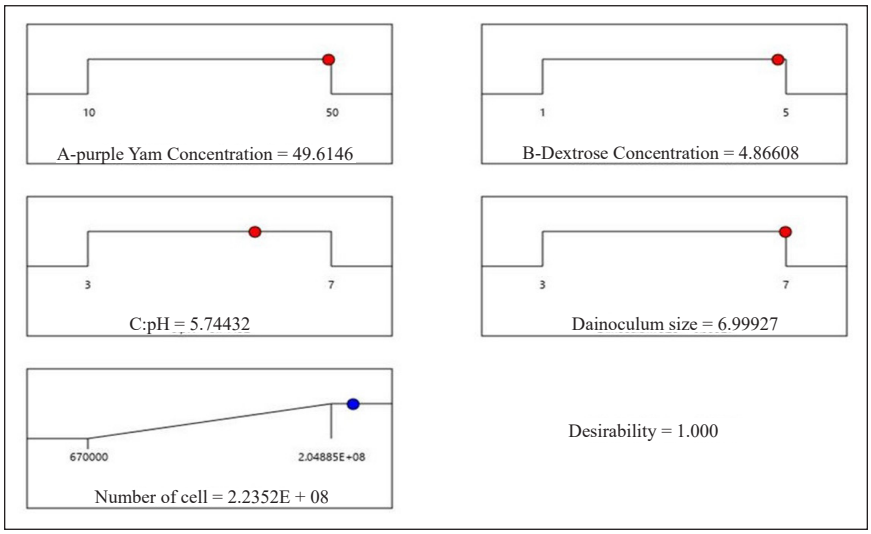

Figure 5. The optimal conditions of the independent variables and the predicted response

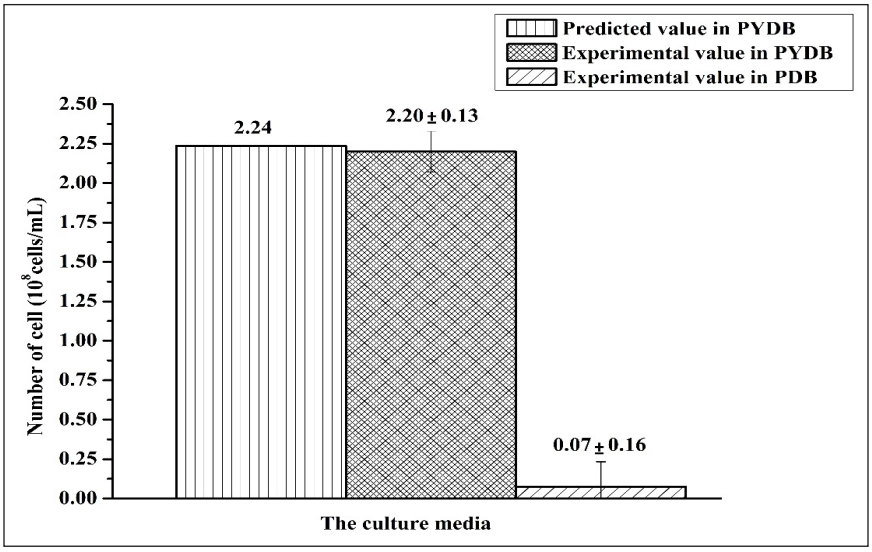

Figure 6. The comparison of the number of cells in purple yam dextrose broth (PYDB) under optimized conditions with the predicted value and the number of cells in potato dextrose broth (PDB) under standard conditions

Growth Profile of *Saccharomyces cerevisiae* in PYDB under the Optimal Conditions Based on the Results of RSM

The growth profile has been used for describing the microbial growth in broth media over time (Ram et al., 2019). The growth profile of *Saccharomyces cerevisiae* in PYDB under optimized culture conditions is shown in Figure 7. The lag phase of *S. cerevisiae* growth was found from the initial incubation period until 6 hours of

incubation. The yeast takes a little time to accommodate itself to its new habitat and environment. The number of *S. cerevisiae* increased exponentially from the sixth hour of incubation to the twenty-fourth hour of incubation, this stage is called the exponential phase or logarithmic phase (log phase). In the late exponential phase, the diauxic shift was observed at the eighteenth hour of incubation when glucose was restricted, and the growth rate of *S. cerevisiae* in PYDB decreased from the eighteenth hour of incubation to the twenty-fourth hour of incubation (the post-diauxic growth period) (Herman, 2002). The highest number of *S. cerevisiae* in PYDB under optimized culture conditions was found at the twenty-fourth hour of incubation, equal to $2.19 \times 10^8 \pm 0.05$ cells/mL (Figure 7). After that, the *S. cerevisiae* growth entered a stationary phase, observing from the constant number of cells. Therefore, the optimal incubation time of *S. cerevisiae* in PYDB under optimized culture conditions for *S. cerevisiae* biomass production was twenty-four hours.

CONCLUSION

The finding from this study concluded that purple yam has a high potential for utilization as alternative culture media in fungal cultivation. The growth of mushroom, mold, and yeast in PYDB media containing 40% purple yam was greater than potato dextrose media. In the same media, the biomass production and growth of *Saccharomyces cerevisiae* under optimized culture conditions were higher than those in PDB under standard conditions. The growth of *S. cerevisiae* in PYDB under optimized conditions was thirty times better than the growth of *S. cerevisiae* in PDB under standard conditions. Taken together,

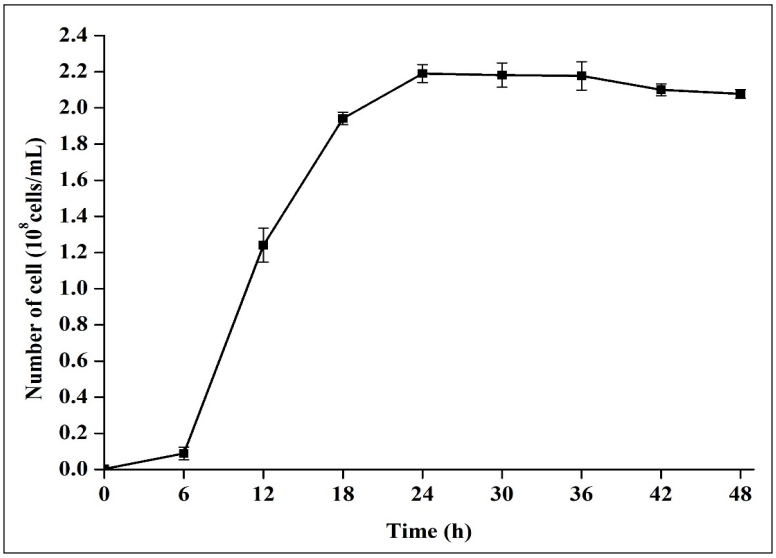

Figure 7. The growth profile of *Saccharomyces cerevisiae* in purple yam dextrose broth under optimized culture conditions

purple yam is a suitable tuber crop and an alternative nutrient source for fungal cultivation. The biomass production of other fungi and their products using purple yam in the media preparation should be further studied.

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Diversity, Relative Abundance, and Functional Genes of Intestinal Microbiota of Tiger Grouper *(Epinephelus fuscoguttatus)* and Asian Seabass *(Lates calcarifer)* Reared in A Semi-Closed Hatchery in Dry and Wet Seasons

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ABSTRACT

Grouper and Asian seabass are among the economically important cultured marine fish in Malaysia. However, fry productions in large scale tend to introduce stress that changes the fish microbiota and increases susceptibility to diseases. Currently, high-throughput sequencing is used to study fish microbiota and their respective gene functions. In this study, the diversity, abundance and functional genes of intestinal microbiota of tiger grouper and Asian seabass that were reared in a semi-closed hatchery during dry and wet seasons. Intestinal samples were collected from tiger grouper and Asian seabass of different sizes before proceeded to DNA extraction. The extracted DNA were then subjected

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to 16S rRNA gene amplicon sequencing using the Illumina Miseq platform targeting V3 and V4 regions for determination of the bacterial diversity, abundance and functional genes in both seasons were investigated. The results revealed that intestinal microbiota of Asian seabass were dominated by the phylum Proteobacteria and order Vibrionales in both seasons. Meanwhile, intestinal microbiome of tiger groupers were shifted from domination of phylum Firmicutes and order Clostridiales in dry season to Proteobacteria and order Lactobacillales in wet season. PICRUSt analysis revealed that the functional genes that were dominantly present were the genes encoded for metabolism, genetic information processing, environmental information processing, cellular process and human diseases. Remarkably, SIMPER analysis showed several potential metagenomics biomarker genes in dry and wet seasons. This study revealed the importance of utilizing amplicon metagenomics approaches in microbiome studies for better identification of the microbial profiling in aquaculture systems.

Keywords: Asian seabass, marine fish hatchery, metagenomics, tiger grouper

INTRODUCTION

Aquaculture has developed rapidly to become one of the most important food industries in the world (Little et al., 2016). In recent years, increased demands from local and export markets for high-value fish species, such as groupers (*Epinephelus* spp.), Asian seabass (*Lates calcarifer*), and snapper (*Lutjanus* spp.) have encouraged hatcheries to produce more fry (Othman et al., 2017). The increasing trend towards developing large-scale production has led to intensive marine aquaculture practices and enhancing vulnerability of fish to disease outbreaks that affects fry production and quality (Ahmad et al., 2019; Ismail et al., 2017; Tarnecki et al., 2017).

A metagenomics study in aquaculture helps to identify pathogens before they cause disease outbreaks in marine fish hatcheries (Martínez-Porchas & Vargas-Albores, 2017). Furthermore, metagenomic analysis has expanded our knowledge by revealing enormous microbial communities, and some unknown microbial diversity, in a variety of environments (Debroas et al., 2009; Hewson et al., 2009). Within the marine fish hatchery ecosystem, the gastrointestinal tract of fish and the holding water possess hugely diversified microbial communities that vary depending on the fish species (Di Maiuta et al., 2013; Wu et al., 2010). They are considered the main potential sources of infection for many fish pathogens (Barkham et al., 2019; Givens et al., 2015; Roeselers et al., 2011). Moreover, studies have indicated that microbial communities are largely influenced by the environmental factors, such as water salinity, seasons, and geographical area surrounding the host (Amal et al., 2010; Dehler et al., 2017a, 2017b; M. Zhang et al., 2016; Wu et al., 2012).

Numerous attempts were made to explore the bacterial microbiome within the fish hatchery ecosystem using the culture-based method. Unfortunately, this method has limitations, as only < 10% of the bacteria could be isolated and cultured under laboratory conditions (Lyons et al., 2015; Tarnecki et al., 2017). On the other hand, next-generation sequencing (NGS) platforms could explore the microbiome communities on unprecedented scale, and allowed identification of both culturable and unculturable bacterial communities within the marine fish hatchery (Wang et al., 2018).

There is scarce information on the effects of seasonal factors on microbiome and metagenome within the tropical marine fish hatchery. Thus, the present study aimed to compare the diversity, relative abundance, and functional genes of intestinal microbiota of tiger grouper and Asian seabass that were cultured in a semi-closed tropical marine fish hatchery in dry and wet seasons.

MATERIALS AND METHODS

Study Site

This study was carried out in a semi-closed tropical marine fish hatchery producing tiger grouper (*Epinephelus fuscoguttatus*) and Asian seabass (*Lates calcarifer*) fry. This hatchery was located nearby the sea at the east coast of Peninsular Malaysia (5.8290° N, 102.5524° E). It was defined as a semi-closed system because the water supply was directly obtained from the nearby open sea throughout the year, was filtered, treated, and aerated before being channelled into the hatchery. Thus, the water quality in the hatchery is influenced by dry and wet seasons.

Fish Samples

Duplicate samples of fish were collected during the dry (July 2018) and wet (November 2018) seasons. One-time random sampling was made in each season involving various sizes and production batches of tiger groupers (n = 9) and Asian seabass (n = 10 - 13).

The sampled tiger groupers and Asian seabass were sedated with tricaine methanesulphonate (MS-222; 50 mg/L) before the total body length and weight of each fish were recorded (Table 1). Immediately, the dissections were performed in a sterile condition, where the intestinal samples of respective fish species were pooled and stored in a sterile falcon tube containing 20 mL of RNAlater™ solution (Thermo Fisher Scientific, MA, USA) for subsequent DNA analysis. All intestinal samples were kept on ice, transported back to the laboratory, and stored at -80°C until further analysis. The fishes were sampled, handled, and sacrificed according to the methods approved by Institutional Animal Care and Use Committee, Universiti Putra Malaysia.

Water Physicochemical and Seasonal Parameters

During each sampling, the water physicochemical parameters such as pH, dissolved oxygen (DO), total dissolved solid (TDS), water temperature (T), conductivity (C), salinity (S), ammonia-nitrogen (NH_3-N),

Table 1

Summary of collected fish and water samples for dry and wet seasons

Season	Fish species	Samples code (pooled samples)	Number of fish	Length Mean ± SD (cm)	Weight Mean ± SD (g)
Dry	Tiger grouper	DTG1	5	16.37 ± 5.46	54.00 ± 44.03
		DTG2	4		
	Asian seabass	DAS1	5	14.27 ± 4.45	41.93 ± 35.12
		DAS2	5		
Wet	Tiger grouper	WTG1	5	20.86 ± 8.73	133.43 ± 158.15
		WTG2	4		
	Asian seabass	WAS1	7	16.13 ± 6.83	60.45 ± 55.07
		WAS2	6		

Note. DTG1 and DTG2 are intestinal samples from tiger grouper for dry season; DAS1 and DAS2 are intestinal samples from Asian seabass for dry season; WTG1 and WTG2 are intestinal samples from tiger grouper for wet season; WAS1 and WAS2 are intestinal samples from Asian seabass for wet season

nitrite (NO_2^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulphate (SO_4^{2-}) were measured and recorded accordingly either by using YSI 556 MPS probe (YSI Incorporated, NY, USA) or DR900 spectrophotometer (Hach Company, Loveland, USA). Parameters for the respective season, such as average rainfall (AR), average temperature (AT), and average humidity (AH) were obtained from the Malaysian Meteorological Department, Ministry of Energy, Science, Technology, Environment and Climate Change, Malaysia.

Isolation of Genomic DNA

All intestinal samples were thawed at room temperature before being washed with sterile phosphate buffered saline solution (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) thrice. The genomic DNA was extracted using Favorprep™ Stool DNA Isolation Mini Kit (Favorgen Biotech Corporation, Changzhi, Taiwan), according to the manufacturer's instructions, with additional treatment of RNase A.

Replicates of genomic DNA of tiger groupers and Asian seabass were prepared. The quantity and purity for the extracted DNA were tested using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA) in 1% agarose gel. All the extracted DNA was stored at -80°C until further processing.

High-Throughput Miseq Illumina Sequencing Platform

Eight DNA samples, consisting of four DNA samples from each dry and wet

season (two from tiger grouper and two from Asian seabass) were sent for sequencing to Novogene Biological Information Technology Co. (Tianjin, China), through Apical Scientific Sdn. Bhd. (Seri Kembangan, Malaysia). The V3-V4 region of 16S rRNA was amplified using 16S rRNA gene PCR primers for classical and next-generation sequencing-based diversity studies, and the Illumina adapter overhang nucleotide were added to gene specific sequences (Klindworth et al., 2013). The 16S rRNA amplicon PCR forward and reverse primers were as follows:

Forward - (5'-TCGTCGGCAGTGTGTATAAGAGACAGCCTACGGGNGGCWGCAG -3'); reverse - (5'-TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC - 3'). The 16S rRNA amplicon sequencing was done through the Illumina Miseq (San Diego, California, U.S.A) platform, resulting in the 250 bp paired-end reads.

16S rRNA Amplicon Sequencing Data Analysis

Paired-end sequences were obtained in .fastq format for all samples. All sequences were further trimmed to remove primer and barcoded sequences using Paired-end adapter trimming (PEAT) (Magoč & Salzberg, 2011). Fastq files were imported into Quantitative Insight into Microbial Ecology (QIIME) software (v1.7.0), and merged accordingly to each respective sample using PEAR (Zhang et al., 2013). The merged sequences were filtered using the fastq_quality_filter script under the fastx_toolkit with q = 20 and p = 70. Chimeric sequences were screened, using UCHIME against the RDP_GOLD v9 database and were removed from the downstream processing (Haas et al., 2011). Sequences shorter than 100 bp or longer than 600 bp were removed along with low quality bases ($Q \leq 33$).

Operational taxonomic units (OTUs) were selected with ≥ 97% similarity using the pick_otus.py script with the usearch_ref method against the Greengenes database (Edgar et al., 2011). OTU table was constructed and validated following the OTU picking. Alpha diversity metrics were calculated at the same sequence depth of minimum reads for observed species, Chao1, and community diversity indices (Shannon and Simpson). Association of OTU for each dry and wet season were displayed by using Venn diagram which were constructed by using R software on "ggplot" packages. Bray-Curtis distant assessment was also measured to estimate the beta diversity for each season (Bray & Curtis, 1957). The computation of Bray-Curtis and PERMANOVA test were done with Paleontological Statistics (PAST) software (v3.11) (Hammer et al., 2001). The Bray-Curtis estimated distances were used to plot principal coordinates analysis (PCoA). Microorganisms that were specifically associated with each sample were characterised using the Linear discriminant analysis effect size (LEfSe), which measured both biological relevance and statistical significance (Segata et al., 2011).

Metagenome Prediction of 16S rRNA Datasets

Functional prediction from the 16S rRNA datasets for all samples were conducted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software (Langille et al., 2013) in the Galaxy server. OTU abundances, resulting against the Greengenes database, act as the input file in BIOM formatted for PICRUSt (v1.1.0). The input file was uploaded into Galaxy Langille Lab (Langille et al., 2013) for 16S rRNA gene copy number normalisation using the normalize_by_copy_number.py script followed by metagenome prediction using the predict_metagenomes.py script, against the KEGG Orthology (KO) database. Similarity Percentage analysis (SIMPER) was conducted to choose top 10 genes that showed higher differential average contributions towards each season. All predicted metagenomes were categorised by their functions using categorize_by_function.py script and collapsed respectively into the level 1 and level 2 gene pathways. The metagenome contribution of taxa to different KEGG Orthologs (KOs) were computed to see the related taxa that contribute to differentiation between dry and wet season using metagenome_contribution.py.

Data Analysis

All environmental parameters consist of physicochemical and seasonal parameters were subjected to Shapiro-Wilk for normality distribution test before t-tests analysis using IBM SPSS (Version 21.0, IBM Corporation, Chicago, IL, USA) to test for significance difference between dry and wet seasons. Alpha diversity indices matrices were also subjected for fitness to a normal distribution by Shapiro-Wilk test and followed with t-test analysis by using IBM SPSS (Version 21.0, IBM Corporation, Chicago, IL, USA) to tests for a significant difference between dry and wet season. Statistical significance was determined at $p < 0.05$.

Data Availability

The 16S rRNA datasets were deposited in the NCBI Sequence Read Archive database under the following BioProject: PRJNA602621 with accession numbers of SRX7616297, SRX7616296, SRX7616295, SRX7616294, SRX7616291, SRX7616290, SRX7616287, and SRX7616286.

RESULTS

Fish and Water Quality Analysis

The average length and weight of tiger grouper and Asian seabass that were collected during the study period are presented in Table 1. The T, S, TDS, C, NH_3-N, NO_3^-, NO_2^-, and SO_4^{2-} were among the physicochemical parameters that showed significant differences ($p < 0.05$) between the dry and wet seasons (Table 2). Other than that, seasonal parameters such as AR and AT also showed significant differences ($p < 0.05$) between the seasons. Among the 14 environmental parameters that were measured, a total of 10 parameters showed significant differences ($p < 0.05$) between both seasons in this hatchery.

Table 2

Environmental parameters of water samples between dry and wet seasons

Seasons	Dry	Wet
T (°C)*	30.77 ± 0.06	29.57 ± 0.11
pH (1 – 14)	7.59 ± 0.13	7.46 ± 0.18
S (ppt)*	27.53 ± 0.03	26.36 ± 0.06
DO (mg/L)	3.84 ± 0.86	5.57 ± 0.06
TDS (g/L)*	27,986.83 ± 13.53	26,873.33 ± 47.18
C (µs/cm)*	43,057.00 ± 21.70	41,341.33 ± 72.34
NH_3-N (mg/L)*	0.18 ± 0.04	0.37 ± 0.01
NO_2^- (mg/L)*	0.01 ± 0.00	0.04 ± 0.01
NO_3^- (mg/L)*	0.83 ± 0.06	0.43 ± 0.06
PO_4^{3-} (mg/L)	0.24 ± 0.01	0.27 ± 0.29
SO_4^{2-} (mg/L)*	2,000.00 ± 0.00	2,500.00 ± 200.00
AR (mm)*	7.43 ± 11.50	22.60 ± 30.80
AT (◦C)*	27.90 ± 0.90	26.40 ± 1.00
AH (%)	80.90 ± 3.40	86.80 ± 5.20

Note. T: water temperature; S: salinity; DO: dissolved oxygen; TDS: total dissolved solid; C: water conductivity; NH_3-N: ammonia-nitrogen; NO_2^-: nitrite; NO_3^-: nitrate; PO_4^{3-}: phosphate; SO_4^{2-}: sulphate; AR: average rainfall; AT: ambient temperature; AH: average humidity. * indicates significant difference ($p < 0.05$) between dry and wet seasons

16S rRNA Sequencing Summary

There were a total of 1,923,874 paired-end reads generated from the Illumina Miseq sequencing. A range of 151,126 - 297,541 clean tags, 124,104 - 280,232 effective tags, and 98,184 - 264,838 taxons tags were obtained across all 16S rRNA gene sequencing samples (Table 3). Based on the 97% similarity cut off, between 941 and 2,100 operational taxonomic units (OTUs) were recorded in the samples.

Analysis of Bacterial Community Structure and Composition in 16S rRNA Datasets Chao1, Shannon and Simpson diversity values varied from 220.40 – 1,547.01, 2.73 – 3.75, and 0.87 - 0.94, respectively (Table 4A). Generally, alpha diversity indicated that Asian seabass had higher contribution in terms of richness and evenness during dry and wet seasons. In Asian seabass, all indices measure of the intestinal samples during the wet season (WAS) were higher compared to dry season (DAS). Meanwhile, for tiger grouper, obvious differences were shown in observed species and Chao1, where intestinal samples during dry seasons (DTG) showed higher measures compared to the

wet season (WTG). All diversity metrics showed significant differences ($p < 0.05$) across Asian seabass and tiger grouper in both seasons (Table 4B). On the other hand, the intestinal samples of tiger grouper from the dry season (DTG) had significantly ($p < 0.05$) higher diversity in Chao1 than the wet season (WTG), but not for the Simpson measure (Table 4C).

A comparison between dry and wet seasons on beta diversity and composition of bacterial OTUs showed significant differences ($p < 0.05$; $p = 0.0023$) (PERMANOVA; $F = 3.458$, $p = 0.001$) (Figure 1A). Principal coordination analysis (PCoA) supported the data, as the cluster of dry season was separated further from wet season (Figure 1B).

Relative abundances of top 10 phyla profiles of bacterial OTUs were showed in Figure 2. All samples were dominated by Firmicutes, Proteobacteria, Fusobacteria, Actinobacteria, Plantomycetes, Bacteroidetes, Verrucomicrobia, Cyanobacteria, SBR1093, and Acidobacteria. Both DAS and WAS were represented mainly by the phyla of Proteobacteria (43.45% and 43.10%), Firmicutes (33.51% and 38.99%), and Fusobacteria (22.71% and 15.05%). In contrast, DTG phyla were dominated by Firmicutes (88.34%), followed by Fusobacteria (17.88%), and Proteobacteria (5.99%). Meanwhile in WTG, Proteobacteria (57.33%) were the most abundant phyla, followed by Firmicutes (32.41%) and Fusobacteria (8.83%).

Clostridiales, Vibrionales, Fusobacteriales, Lactobacillales, Rhodobacterales, Alteromonadales, Aeromonadales, Anthomonadales, Enterobacteriales, and Tericiabacterales were the top 10 order across all samples (Figure 2). DAS was dominated by Vibrionales (41.62%), followed by Clostridiales (32.33%) and Fusobacteriales (22.71%). Meanwhile, WAS was dominated by Rhodobacterales (24.17%), Lactobacillales (23.20%) and Vibrionales (16.25%). For DTG, Clostridiales was dominant at 87.25%, followed by Fusobacteriales (7.06%) and Vibrionales (3.44%). In contrast, WTG was mainly presented by Lactobacillales (25.51%), Vibrionales (19.27%), and Alteromonadales (13.03%).

Additionally, LEfSe analysis showed differential taxa in both Asian seabass (AS) and tiger grouper (TG) samples. Figure 3 shows that the intestinal samples of tiger grouper had more specific taxa than the intestinal samples from Asian seabass, which consisted of Xanthomonadales, Enterobacteriaceae, Enterobacteriales and Bacillales with LDA higher than 3.0. Meanwhile, Staphylococcaceae, Pseudomanadaceae, Bukholderiales, Erysipelotrichales, Erysipelotrichaceae, Erysipelotrichi, Sinobacteraceae, Planococcaceae, Comamonadaceae, Oxalobacteraceae, and Enterococcaceae showed LDA score higher than 2.0. In the AS samples, taxa that dominated with LDA score higher than 3.0 were Plantomycetes, Turicibacterales, and Turicibacteraceae. TM7, Synechococcophycideae,

Table 3

16S rRNA gene sequence summary for all samples

Sample name	Number of paired-end reads	Number of clean tags	Number of effective tags	Number of taxon tags	Number of OTUs
DAS1	250,881	232,016	217,850	206,600	1,124
DAS2	322,790	297,541	280,232	264,838	1,189
DTG1	296,544	266,277	243,617	231,222	1,613
DTG2	266,777	235,594	217,752	208,444	1,647
WAS1	202,608	173,695	126,461	149,327	945
WAS2	198,399	167,687	124,733	146,438	941
WTG1	202,724	166,868	135,286	113,356	2,098
WTG2	183,151	151,126	124,104	98,184	2,100
Mean ± S.D					
DAS	286,835.5 ± 35,954.5	264,778.5 ± 32,762.5	249,041.0 ± 31,191.0	235,719.0 ± 29,119.0	1,156.5 ± 32.5
DTG	281,660.5 ± 14,883.5	250,935.5 ± 15,341.5	230,684.5 ± 12,932.5	219,833.0 ± 11,389.0	1,630.0 ± 17.0
WAS	200,503.5 ± 2,104.5	170,691.0 ± 3,004.0	125,597.0 ± 864.0	147,882.5 ± 1,444.5.0	943.0 ± 2.0
WTG	192,937.5 ± 9,786.5	158,997.0 ± 7,871.0	129,695.0 ± 5,591.0	105,770.0 ± 7,586.0	2,099.0 ± 1.0

Note. SD = standard deviation; Paired-end reads referred to reads obtained from Illumina Miseq platform; Clean tags referred to tags after quality filtering; Effective tags referred to tags after quality filtering and chimera removal; Taxon tags referred to effective tags used for building OTUs to get taxonomic information. DAS1 and DAS2 are intestinal samples from Asian seabass for dry season; DTG1 and DTG2 are intestinal samples from tiger grouper for dry season; WAS1 and WAS2 are intestinal samples from Asian seabass for wet season; WTG1 and WTG2 are intestinal samples from tiger grouper for wet season. DAS is intestinal samples from Asian seabass for dry season; WAS is intestinal samples from Asian seabass for wet season; DTG is intestinal samples from tiger grouper for dry season; WTG is intestinal samples from tiger grouper for wet season

Synechococcales, Synechococcaceae, Phyllobacteriaceae, Plantomycetia, and Plantomycetes were among taxa that has LDA higher than 2.0.

Figure 4 shows the taxa detected between dry and wet season. In TG samples, taxa that mostly present abundantly higher in dry season was only Enterococcaceae, while in wet season, Bacillales, Staphylococcaceae, Planococcaceae, Erysipelotrichi, Erysipelotrichales, Erysipelotrichaceae, Bukholderiales, Comamonadaceae, Enterobacteriales, Oxalobacteraceae, Enterobacteriaceae, Pseudomanadaceae, Xanthomonadales, and Sinobacteraceae were the most abundance.

For AS samples, taxa Synechococcaceae was the only taxa that showed differential abundant in dry season, meanwhile Synechococcales, Synechococcophycideae, Turicibacterales, Turicibacteraceae, Plantomycetes, Plantomycetia, Phyllobacteriaceae, and TM7 were the differential abundant taxa that present in wet season.

Table 4

Alpha diversity metrics for (A) all samples, (B) Asian seabass and (C) tiger grouper samples between dry and wet seasons

(A) Sample name	Observed species	Chao1	Shannon	Simpson
DAS1	842	1,068.66	2.73	0.87
DAS2	830	1,064.00	2.74	0.87
DTG1	1,274	1,526.82	3.07	0.87
DTG2	1,283	1,547.01	3.09	0.89
WAS1	703	819.06	3.73	0.94
WAS2	683	821.78	3.75	0.94
WTG1	203	220.40	2.80	0.90
WTG2	216	229.54	2.85	0.91

(B) Sample	Observed species*	Chao1*	Shannon*	Simpson*
DAS	836.00 ± 6.00	1,066.30 ± 2.30	2.73 ± 0.01	0.87 ± 0.00
WAS	693.00 ± 10.00	820.40 ± 1.30	3.74 ± 0.01	0.94 ± 0.00

Table 4 *(Continued)*

(C)Sample	Observed species*	Chao1*	Shannon*	Simpson*
DTG	1,278.50 ± 4.50	1,536.95 ± 10.10	3.08 ± 0.01	0.88 ± 0.01
WTG	209.50 ± 6.50	224.95 ± 4.60	2.82 ± 0.03	0.91 ± 0.00

Note. DAS1 and DAS2 are intestinal samples from Asian seabass for dry season; DTG1 and DTG2 are intestinal samples from tiger grouper for dry season; WAS1 and WAS2 are intestinal samples from Asian seabass for wet season; WTG1 and WTG2 are intestinal samples from tiger grouper for wet season; DAS is intestinal samples from Asian seabass for dry season; WAS is gut samples from Asian seabass for wet season; DTG is intestinal samples from tiger grouper for dry season; WTG is intestinal samples from tiger grouper for wet season. * indicates significant difference ($p < 0.05$) of the same column only

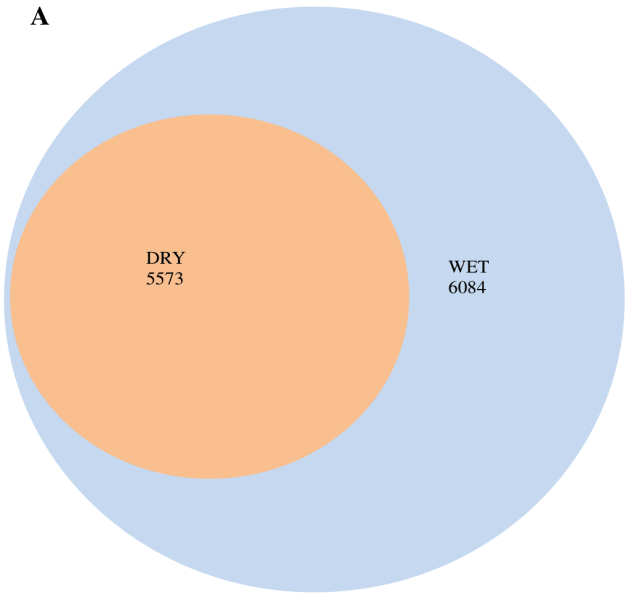


Figure 1. (A) Venn diagram of microbial communities at OTUs level between dry and wet seasons

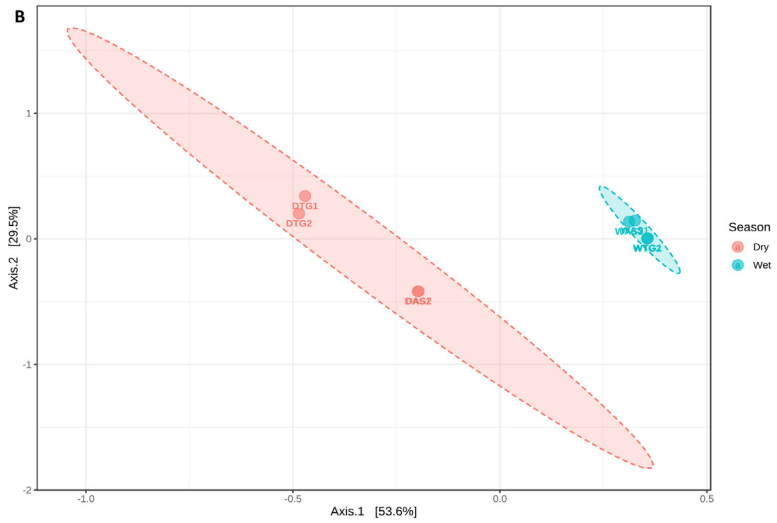
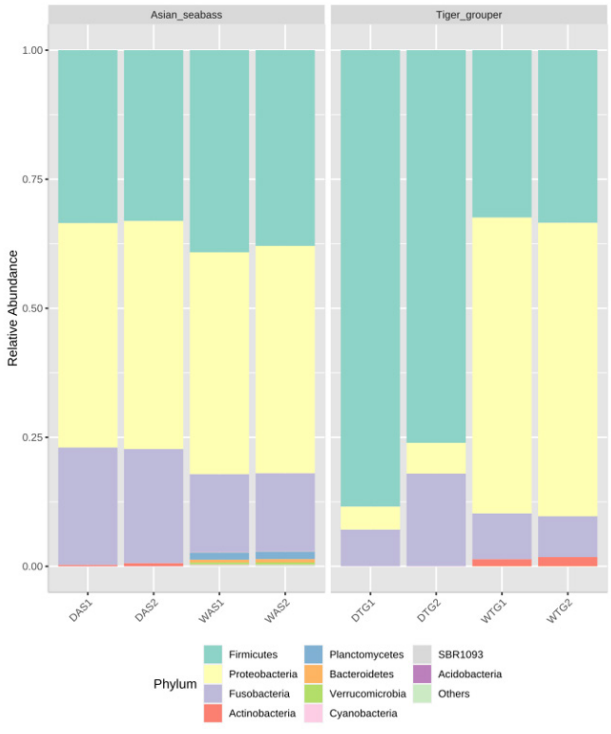
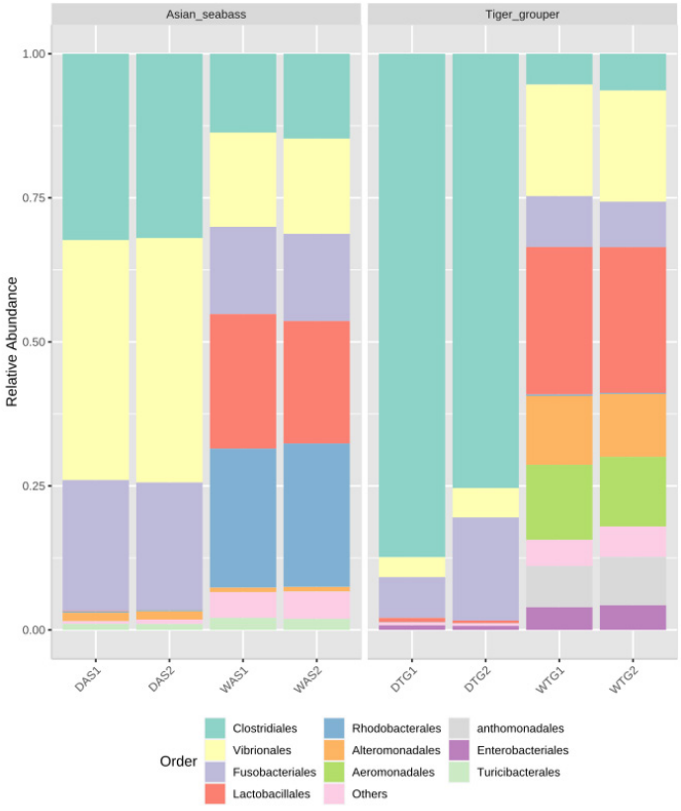


Figure 1(B). Principal coordination analysis (PCoA) between wet and dry seasons

[*Note.* DAS1 and DAS2 are intestinal samples from Asian seabass for dry season; DTG1 and DTG2 are intestinal samples from tiger grouper for dry season; DW1 and DW2 are water samples for dry season; WAS1 and WAS2 are intestinal samples from Asian seabass for wet season; WTG1 and WTG2 are intestinal samples from tiger grouper for wet season; WW1 and WW2 are water samples for wet season]



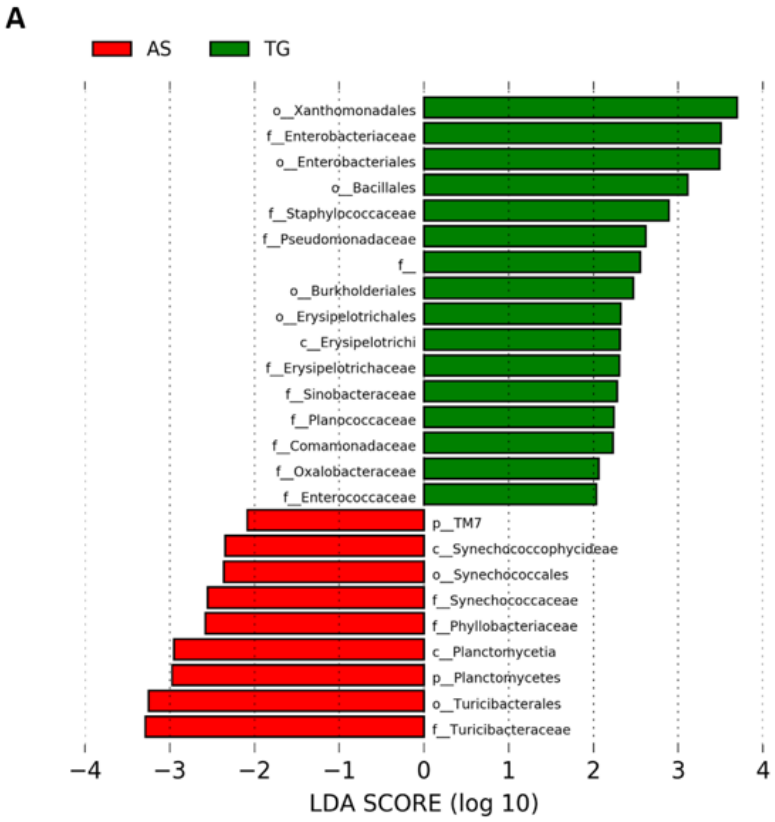
(A)



(B)

Figure 2 (A)&(B). Relative abundance of dominant phyla, and order in each sample resulting from 16S rRNA results between each season

[*Note*. DAS1 and DAS2 are intestinal samples from Asian seabass for dry season; DTG1 and DTG2 are intestinal samples from tiger grouper for dry season; DW1 and DW2 are water samples for dry season; WAS1 and WAS2 are intestinal samples from Asian seabass for wet season; WTG1 and WTG2 are intestinal samples from tiger grouper for wet season; WW1 and WW2 are water samples for wet season]



(A)

Figure 3(A). Differentially abundant bacterial clades detected by LEfSe showing hierarchical structure of the bacterial clades up to order level

[*Note.* AS are intestinal samples from Asian seabass; TG are intestinal samples from tiger grouper]

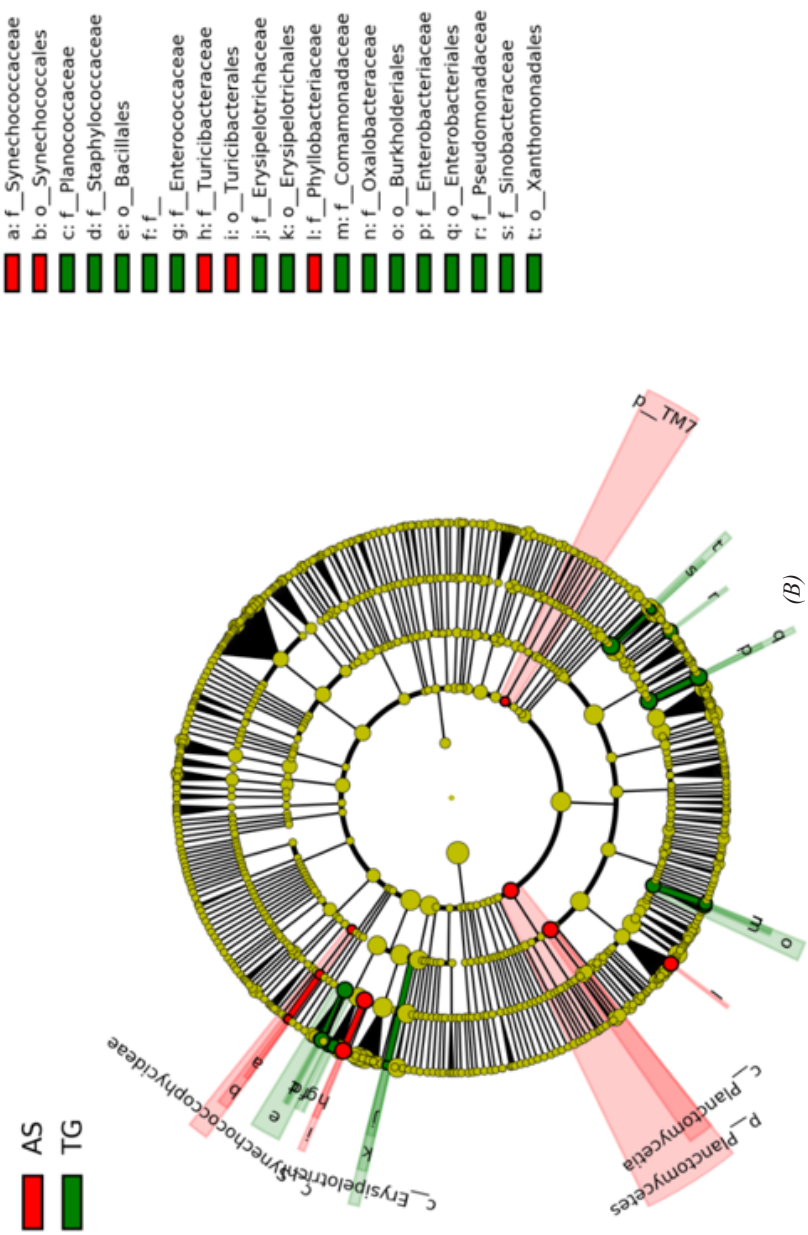
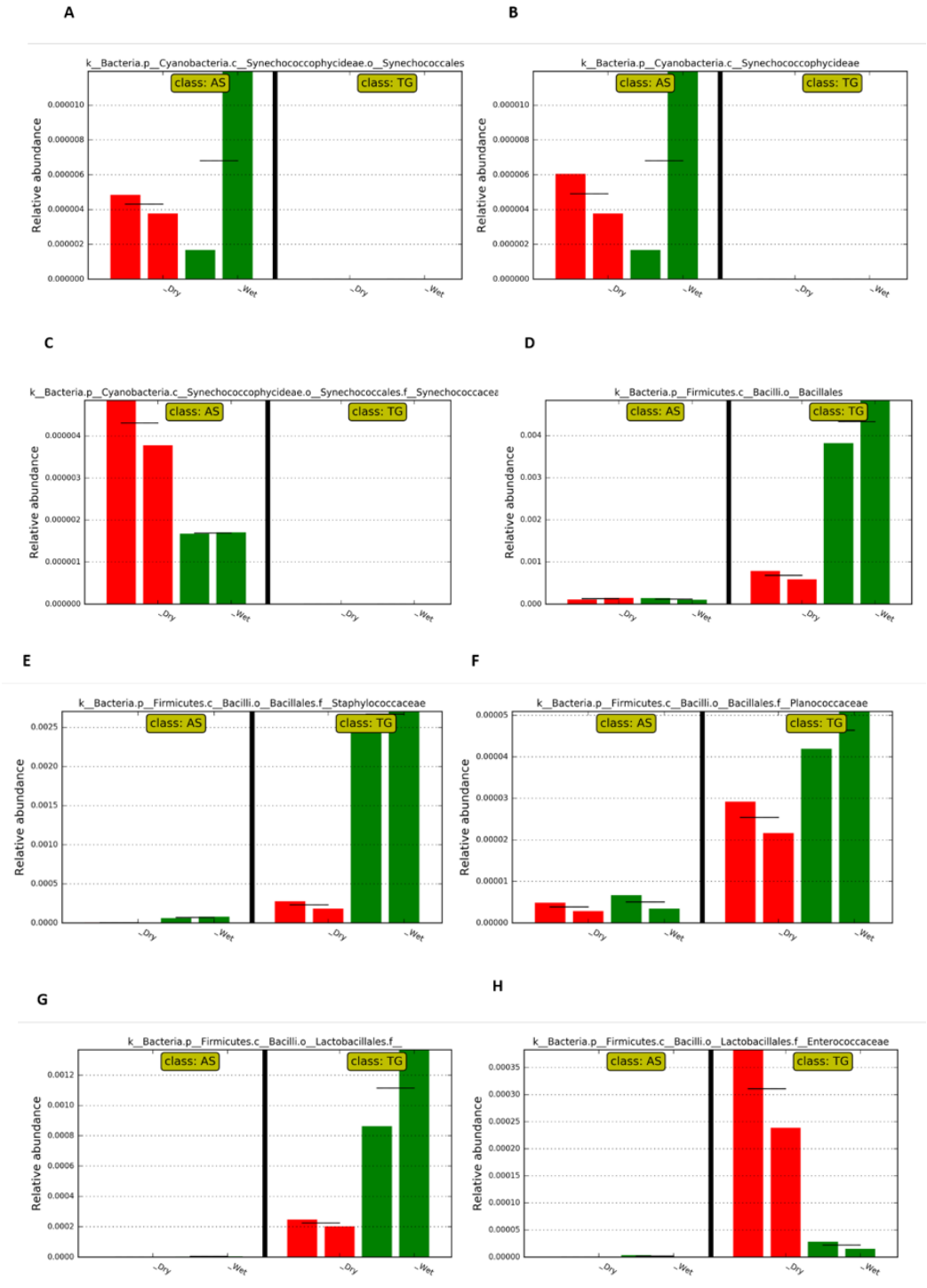


Figure 3(B). Cladogram among Asian seabass and tiger grouper samples showing hierarchical structure of the bacterial clades up to order level [*Note.* AS are intestinal samples from Asian seabass; TG are intestinal samples from tiger grouper]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class: AS
class: TG
Relative abundance
B
k__Bacteria.p__Cyanobacteria.c__Synechococcophycideae
C
k__Bacteria.p__Cyanobacteria.c__Synechococcophycideae.o__Synechococcales.f__Synechococcacea
D
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales
E
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Staphylococcaceae
F
k__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Planococcaceae
G
k__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__
H
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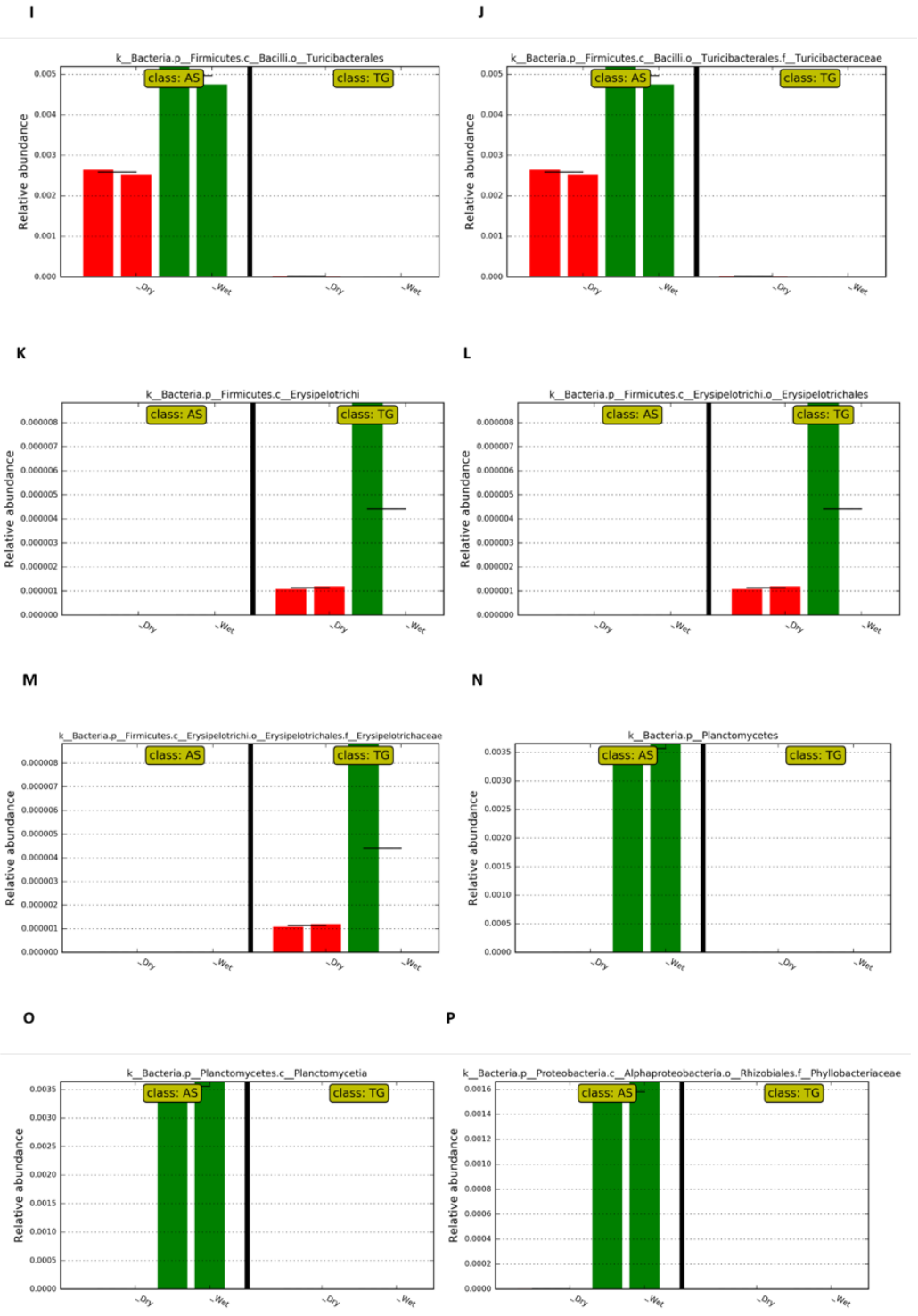
~Dry
~Wet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k__Bacteria.p__Firmicutes.c__Bacilli.o__Turicibacterales
class: AS
class: TG
Relative abundance
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0.004
0.003
0.002
0.001
0.000
~Dry
~Wet
~Dry
~Wet
J
k__Bacteria.p__Firmicutes.c__Bacilli.o__Turicibacterales.f__Turicibacteraceae
class: AS
class: TG
Relative abundance
0.005
0.004
0.003
0.002
0.001
0.000
~Dry
~Wet
~Dry
~Wet
K
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class: AS
class: TG
Relative abundance
0.000008
0.000007
0.000006
0.000005
0.000004
0.000003
0.000002
0.000001
0.000000
~Dry
~Wet
~Dry
~Wet
L
k__Bacteria.p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales
class: AS
class: TG
Relative abundance
0.000008
0.000007
0.000006
0.000005
0.000004
0.000003
0.000002
0.000001
0.000000
~Dry
~Wet
~Dry
~Wet
M
k__Bacteria.p__Firmicutes.c__Erysipelotrichi.o__Erysipelotrichales.f__Erysipelotrichaceae
class: AS
class: TG
Relative abundance
0.000008
0.000007
0.000006
0.000005
0.000004
0.000003
0.000002
0.000001
0.000000
~Dry
~Wet
~Dry
~Wet
N
k__Bacteria.p__Planctomycetes
class: AS
class: TG
Relative abundance
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
~Dry
~Wet
~Dry
~Wet
O
k__Bacteria.p__Planctomycetes.c__Planctomycetia
class: AS
class: TG
Relative abundance
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
~Dry
~Wet
~Dry
~Wet
P
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class: AS
class: TG
Relative abundance
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0.0010
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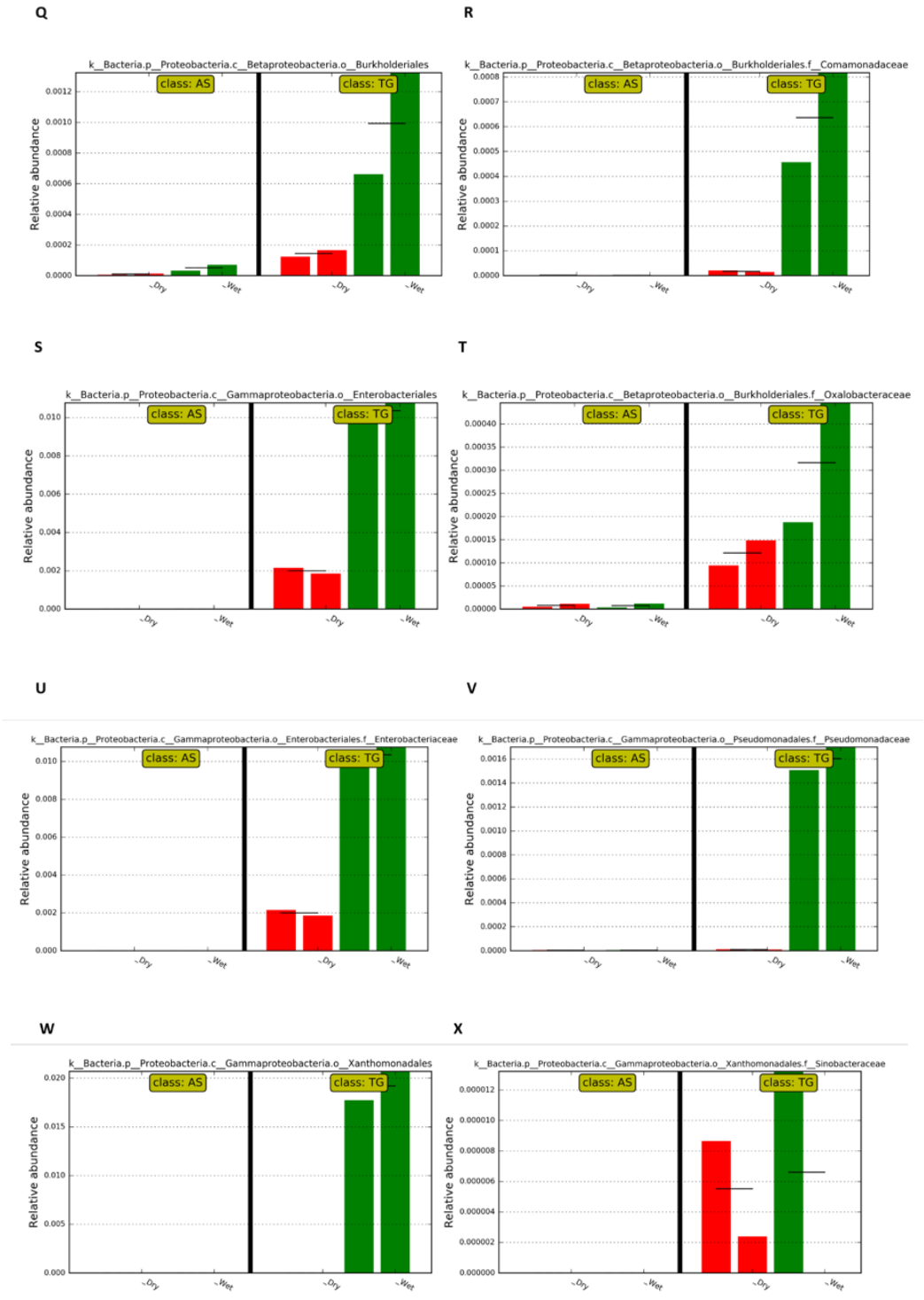
0.0004
0.0002
0.0000
~Dry
~Wet
~Dry
~Wet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class: AS
class: TG
Relative abundance
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0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
~Dry
~Wet
~Dry
~Wet
R
k__Bacteria.p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Comamonadaceae
class: AS
class: TG
Relative abundance
0.0008
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
~Dry
~Wet
~Dry
~Wet
S
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales
class: AS
class: TG
Relative abundance
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0.008
0.006
0.004
0.002
0.000
~Dry
~Wet
~Dry
~Wet
T
k__Bacteria.p__Proteobacteria.c__Betaproteobacteria.o__Burkholderiales.f__Oxalobacteraceae
class: AS
class: TG
Relative abundance
0.00040
0.00035
0.00030
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
~Dry
~Wet
~Dry
~Wet
U
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Enterobacteriales.f__Enterobacteriaceae
class: AS
class: TG
Relative abundance
0.010
0.008
0.006
0.004
0.002
0.000
~Dry
~Wet
~Dry
~Wet
V
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Pseudomonadales.f__Pseudomonadaceae
class: AS
class: TG
Relative abundance
0.0016
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
~Dry
~Wet
~Dry
~Wet
W
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Xanthomonadales
class: AS
class: TG
Relative abundance
0.020
0.015
0.010
0.005
0.000
~Dry
~Wet
~Dry
~Wet
X
k__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Xanthomonadales.f__Sinobacteraceae
class: AS
class: TG
Relative abundance
0.000012
0.000010
0.000008
0.000006
0.000004
0.000002
0.000000
~Dry
~Wet
~Dry
~Wet

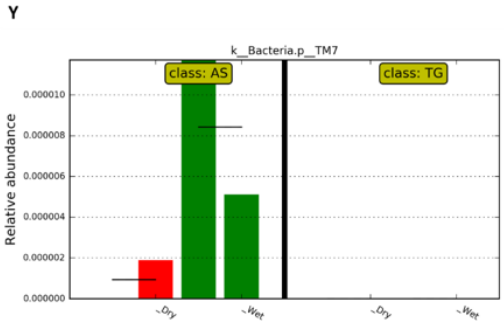


Figure 4. Differentially abundant bacterial clades detected by LEfSe, (A) Synechococcales, (B) Synechococcophycideae, (C) Synechococcacea, (D) Bacillales, (E) Staphylococcaceae, (F) Planococcaceae, (G) Lactobacillales, (H) Enterococcaceae, (I) Turicibacterales, (J) Turicibacteraceae, (K) Erysipelotrichi, (L) Erysipelotrichales, (M) Erysipelotrichaceae, (N) Plantomycetes, (O) Plantomycetia, (P) Phyllobacteriaceae, (Q) Burkholderiales, (R) Comamonadaceae, (S) Enterobacteriales, (T) Oxalobacteraceae, (U) Enterobacteriaceae, (V) Pseudomonaceae, (W) Xanthomonadales, (X) Sinobacteraceae, and (Y) TM7 among Asian seabass and tiger grouper samples in dry and wet seasons

[*Note.* AS are intestinal samples from Asian seabass; TG are intestinal samples from tiger grouper]

Functional Profiles of Microbiotas

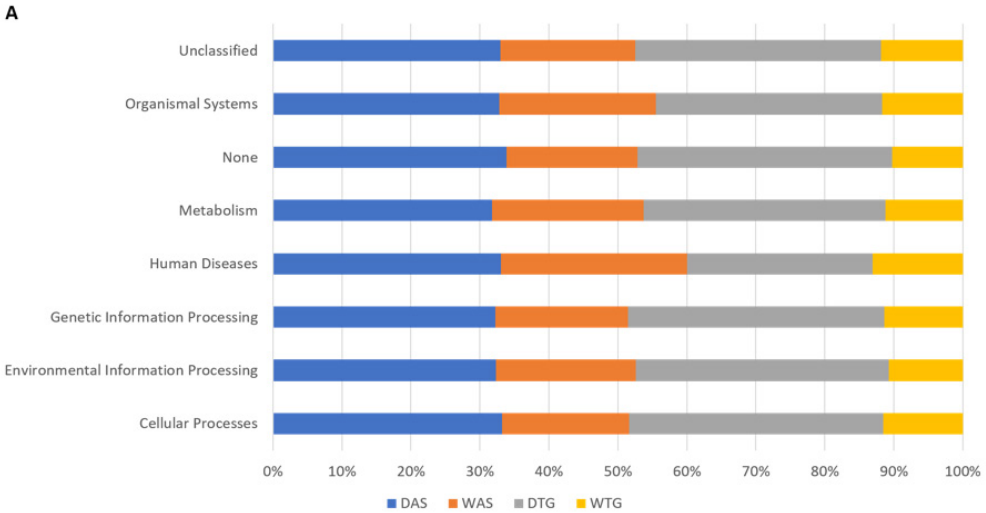
Functional genes of all samples were collapsed into three different levels of KEGG. Level 1 was predominated mainly by metabolism-encoding genes, followed by genes encoding genetic and environmental information processing (Figure 5A). Many genes sequences could not be identified; meanwhile, the lowest functional genes present were under the gene encoding for cellular responses, human diseases and organismal systems. All gene functions in KEGG level 1 were present abundantly in dry season in both tiger grouper and Asian seabass samples.

The KEGG level 2 had a high abundance of genes encoding amino acid, carbohydrate, energy and lipid metabolisms, and genes encoding the metabolism of cofactors and vitamins (Figure 5B). Genes encoding the membrane transport dominated the environmental processing functions, while the genetic information processing encoding for translation also one of abundance gene function. Uniquely, gene function for human disease; cancer was mostly found in sample of Asian seabass during wet season. Generally, most of the gene functions in level 2 were highly present during dry season.

SIMPER analysis revealed that in Asian seabass, K02003, and K02004, which encoded unidentified functional genes, were mostly abundant in the dry season and contributed towards the dissimilarity between the seasons by 61% and 50%, respectively. This was followed by K03406, K09687, K01990, K09686,

K02015, K03091, K07024, and K01448, which were higher in the dry than the wet season, with contribution ranged between 25% and 36% dissimilarity (Figure 6A and Table 5A). Based on the metagenome contribution of KO (Table 6A), family of Peptostreptococcaceae was highly contributed to all KOs of Asian seabass (15% – 27%), while gene K03406 that encodes methyl-accepting chemotaxis protein was associated abundantly with the genus of Vibrio at 19%. All associated taxa were found abundantly in Asian seabass during the dry season.

In the tiger grouper, all KOs were found abundantly in the dry season, with the highest KOs from unidentified functions of K02003 and K02004 (76% and 60%) (Figure 6B). The remaining KOs, K06147, K09687, K01990, K03091, K00936, K09686, K07024, and K03088, contributed to dissimilarity at the rate of between 33% and 46% (Table 5B). Family Peptostreptococcaceae was dominant in KOs of tiger grouper (8% - 14%), while genes K06147 and K03088 were highly influenced by family Clostridiaceae with contributions of 11% and 17%, respectively (Table 6B). All taxa and associated genes were found abundantly throughout the dry season.



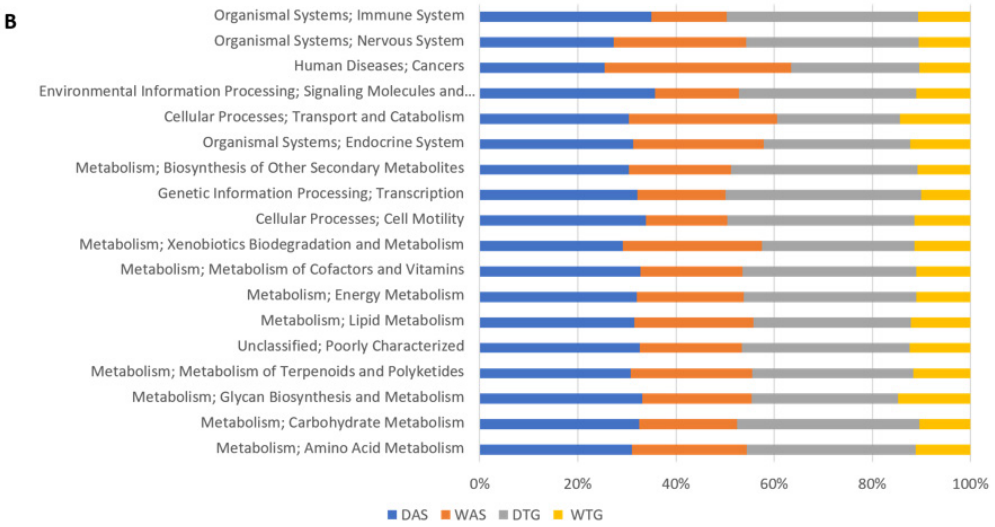
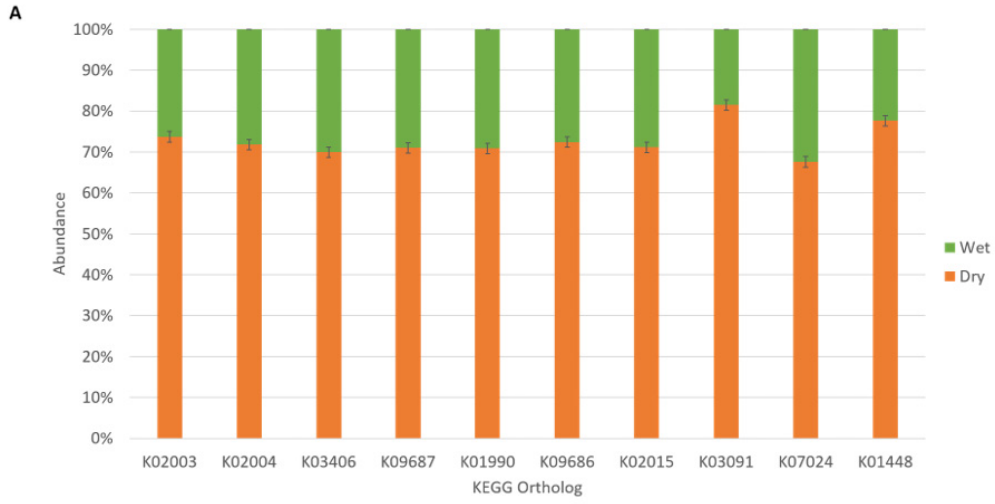


Figure 5. Abundance of genes categorized by functions based on (A) KEGG level 1 and (B) KEGG level 2

[Note. DAS are intestinal samples from Asian seabass for dry season; WAS are intestinal samples from Asian seabass for wet season; DTG are intestinal samples from tiger grouper for dry season; WTG are intestinal samples from tiger grouper for wet season; DW are water samples for dry season; WW are water samples for wet season]



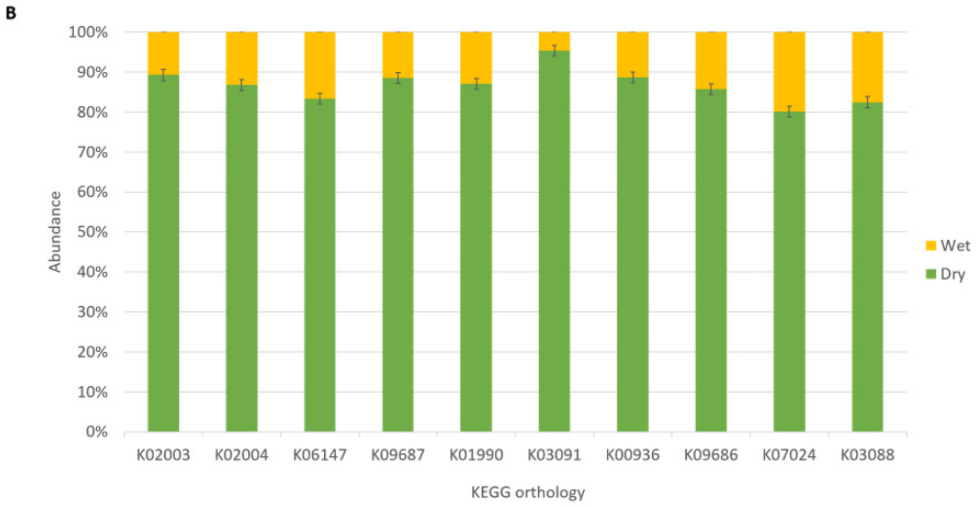


Figure 6. Top 10 of distinct KEGG orthologs in (A) Asian seabass and (B) tiger grouper samples between dry and wet seasons

Table 5

SIMPER analysis of contributions towards the dissimilarity of (A) Asian seabass and (B) tiger grouper samples in comparison between dry and wet seasons

(A) KO	KEGG Description	Average dissimilarity	Contribution (%)
K02003	None	0.218	61
K02004	None	0.176	50
K03406	Methyl-accepting chemotaxis protein	0.129	36
K09687	Antibiotic transport system ATP-binding protein	0.107	30
K01990	ABC-2 type transport system ATP-binding protein	0.103	29
K09686	Antibiotic transport system permease protein	0.100	28
K02015	Iron complex transport system permease protein	0.100	28
K03091	RNA polymerase sporulation-specific sigma factor	0.099	28
K07024	None	0.098	28
K01448	N-acetylmuramoyl-L-alanine amidase [EC:3.5.1.28]	0.088	25

Table 5 *(Continued)*

(B) KO	KEGG Description	Average dissimilarity	Contribution (%)
K02003	None	0.481	76
K02004	None	0.381	60
K06147	ATP-binding cassette, subfamily B, bacterial	0.294	46
K09687	Antibiotic transport system ATP-binding protein	0.284	45
K01990	ABC-2 type transport system ATP-binding protein	0.260	41
K03091	RNA polymerase sporulation-specific sigma factor	0.217	34
K00936	None	0.212	33
K09686	Antibiotic transport system permease protein	0.211	33
K07024	None	0.210	33
K03088	RNA polymerase sigma-70 factor, ECF subfamily	0.208	33

Table 6

Metagenome contribution of KO with their associated taxa of (A) Asian seabass and (B) tiger grouper samples in comparison between dry and wet seasons

(A) KO	KEGG Description	Associated taxa	Taxa contribution (%)	Sample group
K02003	None	Peptostreptococcaceae	27.0	DAS
K02004	None	Peptostreptococcaceae	24.0	DAS
K03406	Methyl-accepting chemotaxis protein	*Vibrio*	19.0	DAS
K09687	Antibiotic transport system ATP-binding protein	Peptostreptococcaceae	25.0	DAS
K01990	ABC-2 type transport system ATP-binding protein	Peptostreptococcaceae	27.0	DAS
K09686	Antibiotic transport system permease protein	Peptostreptococcaceae	25.0	DAS
K02015	Iron complex transport system permease protein	Peptostreptococcaceae	18.0	DAS

Table 6 *(Continued)*

(A) KO	KEGG Description	Associated taxa	Taxa contribution (%)	Sample group
K03091	RNA polymerase sporulation-specific sigma factor	Peptostreptococcaceae	27.0	DAS
K07024	None	Peptostreptococcaceae	15.0	DAS
K01448	N-acetylmuramoyl-L-alanine amidase [EC:3.5.1.28]	Peptostreptococcaceae	27.0	DAS

(B) KO	KEGG Description	Associated taxa	Taxa contribution (%)	Sample group
K02003	None	Peptostreptococcaceae	14.0	DTG
K02004	None	Peptostreptococcaceae	13.0	DTG
K06147	ATP-binding cassette, subfamily B, bacterial	Clostridiaceae	11.0	DTG
K09687	Antibiotic transport system ATP-binding protein	Peptostreptococcaceae	12.0	DTG
K01990	ABC-2 type transport system ATP-binding protein	Peptostreptococcaceae	13.0	DTG
K03091	RNA polymerase sporulation-specific sigma factor	Peptostreptococcaceae	13.0	DTG
K00936	None	Peptostreptococcaceae	11.0	DTG
K09686	Antibiotic transport system permease protein	Peptostreptococcaceae	14.0	DTG
K07024	None	Peptostreptococcaceae	8.0	DTG
K03088	RNA polymerase sigma-70 factor, ECF subfamily	Clostridiaceae	17.0	DTG

DISCUSSION

In this study, the alpha diversity indices demonstrated that bacterial communities in the intestinal samples of tiger grouper and Asian seabass that were reared in a semi-closed tropical marine fish hatchery were greatly influenced by seasons and was significantly higher in the dry season. Therefore, there was a microbiome shift according to the seasons in the hatchery. Overall, the fish intestinal microbiome in

this study was dominated by the phylum of Proteobacteria, Firmicutes, Fusobacteria, and Plantomycetes, and the order of Clostridiales, Vibrionales, Fusobacteriales, Lactobacillales, and Rhodobacterales, as reported in previous studies (Dehler et al., 2017a, 2017b; Huang et al., 2017; Hennersdorf et al., 2016; Sullam et al., 2012).

Both dry and wet seasons share the same dominant phyla in the intestinal microbiome of Asian seabass, which were Proteobacteria, Firmicutes and Fusobacteria, but they were more abundant in the dry season, when water temperature, ambient temperature, and salinity were significantly higher. Indeed, Vibrionales dominated the intestinal microbiota of Asian seabass in both seasons, but significantly higher in dry season, as reported in a previous study (Zarkasi et al., 2014). This proved that *Vibrio* is a normal flora in the fish intestine and marine water, but also possible to cause vibriosis in cultured fish kept in high water temperature of the dry season (Abdullah et al., 2017; Mohamad et al., 2019c). Although there was no vibriosis outbreak in the period of this study period, it was suspected that further increase in the abundance of *Vibrio* might trigger an outbreak in the hatchery if no precaution was taken. During the wet season, Rhodobacterales and Lactobacillales were highly dominant, and the increasing trend of these bacteria had been related to low water temperature (Dang et al., 2008) and high occurrence of lactic acid bacteria that indicate healthy fish gut (Alonso et al., 2019), respectively.

On the other hand, intestinal microbiome of tiger grouper showed that Firmicutes was dominant during dry season, but low in wet season. Meanwhile, Proteobacteria was dominant during wet season, but low in dry season. This microbiome shift suggests that Gram-positive bacteria prefer dry weather condition, while Gram-negative bacteria showed favour towards wet weather condition. In dry season, tiger groupers were highly dominated by Clostridiales, which is similar to the microbiome of bluegill (*Lepomis macrochirus*) during late summer and fall (Ray, 2016). The diversity of bacteria during wet season was also more diverse compared to the dry season, which was believed to be correlated with the decrease of pH and salinity (Roquigny et al., 2020). Unlike Asian seabass, the raising water temperature seemed to inhibit the proliferation of Vibrionales in tiger grouper. Absence of disease outbreak during this study might suggest that there is equilibrium between pathogenic bacteria with the normal bacterial flora communities.

Comparative LEfSe analysis revealed the potential taxa biomarkers based on seasons. In tiger grouper, order Enterobacteriales was present abundantly, and it is capable of reducing nitrate to nitrite thus, is widely used in numerous applications including biocontrol in agriculture, control of infectious diseases, anticancer agents, and bioremediation (Octavia & Lan, 2014). They were found in the wet season, when the nitrate level was abundant. Staphylococcaceae is one of the members of Bacillales order which is

abundant during wet season. This bacterial family was mainly present on the skin and mucous membranes of animals, but their pathogenicity and infection mechanism (genera *Staphylococcus*) were considered threats due to its resistance towards antibiotics (Naimi et al., 2003). Abundant of this family during wet season showed that they favoured wet condition when water temperature and salinity were lower, while further drop in temperature might increase these bacterial abundances that can lead to foodborne disease due to microbial contamination. Order Lactobacillales was the members of lactic acid bacteria that produce lactic acid at the end of the carbohydrate metabolism and commonly used as probiotics in aquaculture (Walter, 2008). Higher abundance of these bacteria in the tiger grouper's gut suggests that wet season might trigger their abundance, thus stimulating immune response and improving disease resistance in the fish. Moreover, recently Gao et al. (2020) reported that class Erysipelotrichi, order Erysipelotrichales and family Erysipelotrichaeae were one of the newest microbes in gut of carnivorous fish. Only Enterococcaceae was found abundant in dry season and this bacterial taxon is a common inhabitant in the gastrointestinal tract of marine fish (Dehler et al., 2017a, 2017b). This proved that the intestinal microbiota of fish, especially tiger grouper was affected by the seasonal changes. In Asian seabass, Synechococcaceae was the only taxon that differentiated dry and wet season, where this taxon mainly found during dry season. Most of the potential taxa biomarker from tiger grouper and Asian seabass were influenced by the physicochemical parameters, which suggested that the intestinal microbiome of the fish was associated with the water quality too.

Analysis on microbial functional genes revealed that the genes were mainly associated with metabolism, genetic and environmental information processing, and membrane transport, similar to the previous studies (Abia et al., 2018; P. Huang et al., 2018). Methyl-accepting chemotaxis protein (MCP) (K03406) was present in Asian seabass, and was associated with the genus *Vibrio*, which is known for its pathogenicity and disease outbreaks (Amalina et al., 2019; Mohamad et al., 2019a, 2019b). MCP is a sensory transducer that controls exopolysaccharides (EPS) production (Xu et al., 2011). In biofilms, EPS consists of polysaccharide, protein, and nucleic acid that provides structure and strength. Flowing water increases EPS production, resulting in overexpression of cell motility and bacterial chemotaxis (X. Zhang et al., 2019). K09687 and K01990 are closely related to the ABC-2 type transport system ATP-binding protein in antibiotic resistance gene, where these genes encode for ABC transporter protein (Fuellen et al., 2005). In Asian seabass and tiger grouper, most KOs were associated with family Peptostreptococcaceae, a member of allochthonous and autochthonous microbiota and anaerobic bacteria (Ringø et al., 1995). It showed higher contribution during dry season, indicating that the family Peptostreptococcaceae was a natural

part of the intestinal microbiota in Asian seabass and tiger grouper that contribute in regulating internal process of fish species in dry season.

Dissimilar gene contribution in tiger grouper was dominated by environmental information processing (Qu et al., 2008), which is important to fish and ecosystem health (X. Zhang et al., 2019). Thus, family Clostridiaceae was abundant in the healthy fish (de Bruijn et al., 2018) and the presence of marker genes associated with taxa Clostridiaceae throughout the dry season indicates that the tiger grouper was in healthy condition in the dry season. In general, gene functions in Asian seabass and tiger grouper were relatively higher during dry season, which indicate that dry season has more effect on the expression of the gene and supports the finding of dissimilar gene contribution where most of the taxa associated with the KOs were originated in the dry seasons.

CONCLUSION

In this study, the intestinal microbiota of tiger grouper and Asian seabass were influenced by dry and wet season in this semi-closed tropical marine fish hatchery. Moreover, it also showed that amplicon metagenomics analysis could provide useful data to predict and control possible bacterial disease outbreaks in the hatchery based on the intestinal microbiota of the fish. It is recommended for future study to increase the sample size for metagenomics analyses, while the investigation on unclassified taxa and its function in this study should be conducted for more understanding of the fish intestinal microbiome.

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The Orchid Flora of Gunung Ledang (Mount Ophir), Malaysia - 120 Years after Ridley

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ABSTRACT

A comprehensive assessment on the orchid flora of Gunung Ledang, Johor, Malaysia was carried out from 2012 to 2018 with the aim to re-evaluate the presence of orchid species listed by Ridley in his "Journal of the Straits Branch of the Royal Asiatic Society 35:1–28", published in 1901, after more than 100 years. The relevant account for comparison is also listed, noting that Ridley's historical collections were for the isolated group of hills commonly known as Gunung Ledang (Mount Ophir), while the collated item in Orchidaceae is part of catalogues for the whole of Peninsular Malaysia. After Ridley, no account on the orchid flora of Gunung Ledang has been properly given, particularly from the uppermost peak of the mountain, where many interesting plants and orchids are to be found there. This study identified 26 species or 67% were the same as those recorded by Ridley (1901), and 65 species or 83% of Turner (1995) checklist of 270 species of orchids for the state of Malacca and Johor, including the common and widespread species to Peninsular Malaysia. By contribution, this paper provides an updated account on the diversity of orchids in Gunung Ledang, listing 122 species of orchids, of which eight are endemic to Peninsular Malaysia, two are hyper-endemic known only from Gunung Ledang, and 30 were recognised as new records. A comparison table of the

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current findings against Ridley (1901) and Turner (1995) is provided which shows only 16 species were the same in all three studies.

Keywords: Diversity, Gunung Ledang, H. N. Ridley, I. M. Turner, Mount Ophir, Orchidaceae

INTRODUCTION

Gunung Ledang, or historically known as the fabled Mount Ophir is an isolated mountain range consisted of several main peaks, with the highest is Puncak Gunung Ledang that stands at 1267 m above sea level. The mountain range is accessible both from Asahan to the north and from Tangkak to the east, as Gunung Ledang is located within the border of Malacca and Johor.

Knowledge on the orchids of Gunung Ledang was first published by Ridley in 1901, and his records have served as a prominent reference on orchid species diversity of that high isolated mountain. Before Ridley, Gunung Ledang has been visited and explored by Cuming and Lobb (Ridley, 1901) who have made extensive orchid collections and brought down a number of dried specimens as well, yet no account of their collections nor indeed of their expeditions appear to be ever published.

Ridley identified 39 species mostly collected from the uppermost part of the range at the elevation of about 900–1200 m, and only referring to a few of rarer species at the lower levels. Most of Ridley's account consisted of species he collected from the northwest part of the mountain, ascending to the top of the highest peak which is Puncak Gunung Ledang from Malacca *via* Bukit Besar and Lubuk Kedondong, where the mountain is accessible with safety in the early days.

Ridley explored and ascended to the main peak of Gunung Ledang from Malacca *via* two routes. The first route started from the densely wooded Bukit Besar where the ascent commences steeply towards Padang Batu at about 823 m elevation, a large sloping rock-face covered in part with thick grass and sedges, among which grow gnarled montane trees such as *Baeckia*, *Leptospermum*, and *Podocarpus*, with orchids such as *Arundina graminifolia*, *Spathoglottis aurea*, and *Paphiopedilum barbatum* heavily populated the damp spots. However, the population of *S. aurea* in Gunung Ledang seems to be rare nowadays. He then marched through Gunung Tunduk, a large bare rock area which a good view of Malacca is obtainable, before not far off descended into a damp valley, and continued by a stiff steep climb to reach the extreme peak of Gunung Ledang. The second route taken by Ridley was *via* a lower peak of the range known as Gunung Mering, where he ascended with Mr. Hervey in 1892 from Lubuk Kedondong. Ridley reported that Gunung Mering is a less visited peak which claimed by local people as inaccessible, even though the ascent proved to be just merely stiff climbs that in parts require one to cross some smooth steep rocks. About halfway up Gunung Mering, they arrived at a point across Padang Batu, a stone field with a cascading stream and were surrounded by cliffs which were covered with forest. At

present day, both Ridley's historical routes he ascended from Malacca *via* Lubuk Kedondong are now known as the 'Asahan Trail', named after a small town in Jasin that is located within the border of three states. Thus, from now onwards throughout this manuscript, Ridley's historical routes will be consistently cited as Asahan Trail, referring to the same routes taken but with new designation.

During Ridley's day, large part of Gunung Ledang was left unvisited, particularly to the east and south parts of the mountain range that lies within the state of Johor, very likely due to inaccessibility and safety reasons. Nowadays, Gunung Ledang is accessible from Tangkak in the southeast and Jementah in the northwest. The route from Tangkak *via* Sagil is popular among avid hikers, as ascending the Lagenda Trail to the top of Gunung Ledang is challenging but less arduous in comparison to Asahan. At the beginning of the trail, the route is quite treacherous with protruding tree roots, huge rocky boulders, and dense forest canopy. The ascensions commence starting from Batu Orkid towards Bukit Botak, where at some points, requiring one to scale up using ladders and ropes. From Bukit Botak towards Anjung Mahligai (1061 m above sea level), there is a clearing where intermittently covered in mist during the day, the surrounding is beautiful and the summit of Gunung Ledang is within sight. In contrast, the route from Jementah over Ulu Jementah Trail is seldom visited due to the long-winded mountain ridge and extremely steep ascent along the way through several lower peaks such as from Puncak Jementah (945 m above sea level) to Gunung Mahligai (1236 m above sea level) before summiting Gunung Ledang. Water supply is also scarce; a stream with small running water is only accessible at the first 2 km from the trail entrance, and next possible water sources will come from small creeks running through rocks. Most part of the trail is covered in dense lowland and hill forest canopy, untouched, and a best place for the botanical study of many plant groups. No account on the orchid flora from this part of Gunung Ledang has ever been published, thus the accomplishment in preparing the updates will augment the fundamental knowledge that has been firmly set up by Ridley.

The relevant account for comparison by Turner (1995) is also listed, noting that Ridley's historical collections were for the isolated group of hills commonly known as Gunung Ledang, while Turner's item in Orchidaceae is part of his catalogues for the whole of Peninsular Malaysia. In 1995, Turner published his checklist "A Catalogue of the Vascular Plants of Malaya" (in The Gardens' Bulletin Singapore 47:2), an encyclopaedic compilation from his assiduous examination on the herbarium specimens holding of the Singapore Botanical Garden's Herbarium (SING), the Royal Botanic Gardens at Kew (K), and local herbaria in the Forest Research Institute of Malaysia (KEP), University of Malaya (KLU), Biology Department, Universiti Putra Malaysia (UPM), and Universiti Kebangsaan Malaysia (UKMB).

In his checklist, Turner listed 129 species of orchids specifically known from Malacca and Johor, with an additional of 141 species recognized as common and widespread throughout Peninsular Malaysia. He mentioned two hyper-endemic species known only from Gunung Ledang, *Hetaeria elegans* Ridl., which Ridley discovered from Gunung Tunduk and described it in 1908; and *Anoectochilus burmannicus* Rolfe, which is only known from one locality in the Malay Peninsula.

MATERIALS AND METHODS

Ridley's historical routes in Asahan were revisited, with two new routes accessible from Tangkak and Jementah were visited in this study (Figure 1). Seven forest trails were explored and assessed which are, (i) Asahan Trail *via* Lubuk Kedondong, (ii) Asahan Trail *via* Dataran Damai Waterfall (Gunung Mering), (iii) Lagenda Trail *via* Batu Orkid, (iv) Ayer Panas Trail *via* Kolam Gajah, (v) Ulu Jementah Trail *via* Jeram Tinggi, (vi) Gunung Mahligai, and (vii) Gunung Ledang. The collections were made based on convenient sampling method along the seven forest trails from base of the foothill ascent to the peak of Gunung Ledang and *vice versa*. Observations were also made along the tarred road starting from the Taman Hutan Lagenda Park Office towards the Telekom Tower, which is located 500 m from the main peak of Gunung Ledang.

The specimens were identified using the morphological characters described, and the identification keys prepared by Comber (2001), Go et al. (2015), Holttum (1964), Ridley (1907, 1924), Seidenfaden and Smitinand (1959) as well as Seidenfaden and

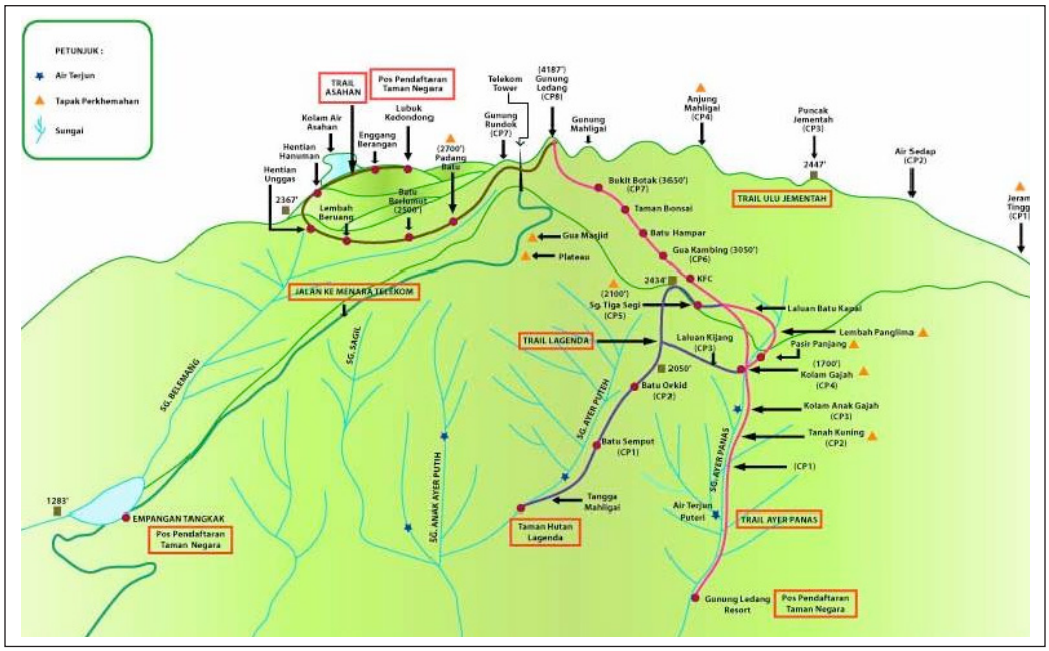

Figure 1. Trails in Gunung Ledang. Map courtesy of Johor National Park Corporation

Wood (1992). The current accepted species name will be validated through the updated online database - Kew World Checklist of Selected Plant Families (WCSP) (2020), and the checklist made available by Ong et al. (2017).

Observations on the vegetative and reproductive macromorphological characteristics of species under investigation would be conducted in the fields. The geographical, ecological, and geological attributes would also be recorded. For detail micromorphological characteristics of selected taxa, specimens would be examined under field and stereo microscopes. Most of the plant specimens would be documented in the form of photographs, and collection of living specimens was to be circumvented in any way possible, as an approach for species as well as habitat preservation and conservation. Preserved herbarium specimens and spirit materials of the taxa collected would be deposited in Herbarium of School of Biological Sciences, Universiti Sains Malaysia (USM). Living collections for germplasm studies and *exsitu* conservation purposes would be cultivated in Taman Flora, School of Biological Sciences, USM.

RESULTS

From this study, a total of 104 orchid species from 62 genera have been identified from Gunung Ledang, of which five are endemic to Peninsular Malaysia, 30 were recognised as new records to the locality, and one undetermined species. The list also serves for comparison with the works by Ridley and by Turner in Malacca and Johor (Table 1).

Table 1
Species list: Comparison of orchid species found in Gunung Ledang since Ridley (1901)

No.	Species	Ridley (1901)	Turner (1995)	Current study
1	*Acriopsis liliifolia* (J.Kōenig) Ormerod var. *liliifolia*		√	√
2	*Aerides odorata* Lour.		√	√
3	*Agrostophyllum stipulatum* (Griff.) Schltr. subsp. *stipulatum*	√		√
4	*Anoectochilus albolineatus* C.S.P.Parish & Rchb.f.		√	√
5	*Anoectochilus geniculatus* Ridl.	√		
6	***Anoectochilus burmannicus* Rolfe		√	
7	*Anoectochilus* sp.			√
8	*Apostasia latifolia* Rolfe	√	√	√
9	*Apostasia nuda* R.Br. in N.Wallich	√	√	√
10	*Apostasia wallichii* R.Br. in N.Wallich			√
11	*Appendicula anceps* Blume		√	√
12	*Appendicula cornuta* Blume		√	√
13	*Appendicula reflexa* Blume		√	√
14	*Arundina graminifolia* (D.Don) Hochr.	√	√	√
15	*Bromheadia aporoides* Rchb.f.	√		
16	*Bromheadia alticola* Ridl.	√	√	
17	*Bromheadia brevifolia* Ridl.		√	
18	*Bromheadia finlaysoniana* (Lindl.) Miq.		√	√
19	**Bromheadia pungens* Ridl.	√	√	√
20	**Bromheadia rupestris* Ridl.	√	√	√
21	*Bromheadia truncata* Seidenf.		√	√
22	*Bulbophyllum clandestinum* Lindl.		√	√

Table 1 (*continue*)

No.	Species	Ridley (1901)	Turner (1995)	Current study
22	*Bulbophyllum clandestinum* Lindl.		√	√
23	*Bulbophyllum elevatopunctatum* J.J.Sm.			√
24	*Bulbophyllum fenestratum* J.J.Sm.			√
25	*Bulbophyllum gracillimum* (Rolfe) Rolfe		√	√
26	*Bulbophyllum purpurascens* Teijism. & Binn.	√	√	√
27	*Bulbophyllum pustulatum* Ridl.		√	
28	*Bulbophyllum striatellum* Ridl.		√	√
29	*Bulbophyllum uniflorum* (Blume) Hassk.		√	√
30	*Bulbophyllum vaginatum* (Lindl.) Rchb.f. in W.G.Walpers	√	√	√
31	*Campanulorchis pellipes* (Rchb.f. ex Hook.f.) Y.P.Ng & P.J.Cribb	√	√	√
32	*Ceratostylis ampullacea* Kraenzl.		√	√
33	*Ceratostylis gracilis* Blume	√		
34	*Ceratostylis subulata* Blume		√	√
35	*Calanthe angustifolia* (Blume) Lindl.	√		√
36	*Claderia viridiflora* Hook.f.	√	√	√
37	*Cleisostoma suffusum* (Ridl.) Garay		√	
38	**Coelogyne anceps* Hook.f.			√
39	*Coelogyne cumingii* Lindl.	√	√	
40	**Coelogyne kaliana* P.J.Cribb			√
41	*Coelogyne testacea* Lindl.		√	√
42	*Coelogyne tomentosa* Lindl.	√		√
43	*Corybas carinatus* (J.J.Sm.) Schltr.		√	√
44	*Corymborkis veratrifolia* (Reinw.) Blume		√	√
45	*Crepidium calophyllum* (Rchb.f.) Szlach.		√	√
46	*Cryptostylis arachnites* (Blume) Hassk. in C.L.Blume		√	√
47	*Cylindrolobus nutans* (Lindl.) J.J.Wood	√	√	√
48	*Cymbidium finlaysonianum* Lindl.			√
49	*Dendrobium angustifolium* (Blume) Lindl.		√	√
50	*Dendrobium convexum* (Blume) Lindl.		√	√
51	*Dendrobium crumenatum* Sw.		√	√
52	*Dendrobium derryi* Ridl.			√
53	*Dendrobium geminatum* (Blume) Lindl.			√
54	*Dendrobium indivisum* (Blume) Miq.		√	√
55	**Dendrobium kelsallii* Ridl.	√		
56	*Dendrobium lamellatum* (Blume) Lindl.			√
57	*Dendrobium leonis* (Lindl.) Rchb.f. in W.G.Walpers		√	√
58	*Dendrobium longipes* Hook.f.			√
59	*Dendrobium macropodum* Hook.f.			√
60	*Dendrobium mannii* Ridl.		√	
61	*Dendrobium metachilinum* Rchb.f.		√	√
62	*Dendrobium pachyglossum* E.C.Parish & Rchb.f.			√
63	*Dendrobium uniflorum* Griff.	√	√	√
64	*Dendrobium villosulum* Wall. ex Lindl.	√	√	√
65	*Dendrochilun linearifolium* Hook.f.	√		√
66	*Dendrochilum longifolium* Rchb.f.			√
67	*Dipodium conduplicatum* J.J.Sm.			√
68	*Erythrodes latifolia* Blume			√
69	*Erythrorchis altissima* (Blume) Blume			√
70	*Galeola nudifolia* Lour.		√	√
71	*Gastrodia javanica* (Blume) Lindl.			√
72	*Geodorum densiflorum* (Lam.) Schltr.		√	√
73	*Goodyera rubicunda* (Blume) Lindl.		√	√
74	*Goodyera viridiflora* (Blume) Blume			√

Table 1 (*continue*)

No.	Species	Ridley (1901)	Turner (1995)	Current study
75	*Grammatophyllum speciosum* Blume		√	√
76	*Habenaria rhodocheila* Hance			√
77	*Hetaeria elata* Hook.f.	√		√
78	***Hetaeria elegans* Ridl.	√	√	
79	*Hylophila mollis* Lindl.		√	√
80	*Lecanorchis malaccensis* Ridl.		√	√
81	*Liparis barbata* Lindl.		√	√
82	*Liparis elegans* Lindl.	√	√	√
83	*Liparis maingayi* (Hook.f.) Ridl.	√	√	
84	*Liparis viridicallus* Holttum			√
85	*Luisia* sp.			√
86	*Macodes petola* (Blume) Lindl.	√		√
87	*Neuwiedia griffithii* Rchb.f.		√	√
88	*Neuwiedia veratrifolia* Blume			√
89	**Oberonia bertoldii* King & Pantl.		√	√
90	*Paphiopedilum barbatum* (Lindl.) Pfitzer	√	√	√
91	*Peristylus maingayi* (King & Pantl.) J.J.Wood & Ormerod		√	√
92	*Peristylus monticola* (Ridl.) Seidenf.	√	√	√
93	*Phalaenopsis deliciosa* Rchb.f.			√
94	*Phalaenopsis fuscata* Rchb.f.			√
95	*Pholidota carnea* (Blume) Lindl. var. *carnea*			√
96	**Pinalia atrovinosa* (Carr) Schuit.		√	
97	*Pinalia bractescens* (Lindl.) Kuntze		√	√
98	*Platanthera angustata* (Blume) Lindl.	√		√
99	*Plocoglottis javanica* Blume		√	√
100	*Podochilus microphyllus* Lindl.	√	√	√
101	*Pomatocalpa diffusum* Breda		√	√
102	*Renanthera histrionica* Rchb.f.	√		√
103	*Rhynchostylis* sp.			√
104	*Spathoglottis aurea* Lindl.	√	√	
105	*Spathoglottis plicata* Blume		√	√
106	*Stichorkis gibbosa* (Finet) J.J.Wood		√	√
107	*Strongyleria pannea* (Lindl.) Schuit.		√	√
108	*Tainia maingayi* Hook.f.	√		
109	*Thecopus maingayi* (Hook.f.) Seidenf.		√	√
110	*Tainia speciosa* Blume	√	√	√
111	*Thrixspermum* sp.			√
112	*Trichotosia ferox* Blume		√	√
113	*Trichotosia gracilis* (Hook.f.) Kraenzl.		√	√
114	*Trichotosia pauciflora* Blume	√	√	
115	*Trichotosia poculata* (Ridl.) Kraenzl.		√	√
116	*Trichotosia velutina* (Lodd. ex Lindl.) Kraenzl.	√	√	
117	*Trichotosia vestita* (Wall. ex Lindl.) Kraenzl.	√	√	
118	*Tropidia angulosa* (Lindl.) Blume			√
119	*Tropidia curculigoides* Lindl.	√	√	√
120	*Vanilla griffithii* Rchb.f.		√	√
121	*Zeuxine affinis* (Lindl.) Benth. ex Hook.f.			√
122	*Zeuxine gracilis* (Breda) Blume			√
	Total	**39**	**78**	**104**

Note.
√ Present
* Endemic to Peninsular Malaysia as reported by Turner (1995) and Ong et al. (2017)
** Hyper-endemic species to Gunung Ledang as reported by Turner (1995)

The finding has identified 26 species or 67% were the same as those recorded by Ridley (1901), and 65 species or 83% of Turner (1995) checklist of 270 species of orchids for Johor, Malacca, and the whole of Peninsular Malaysia. Only 16 species were the same in all three studies, and 18 species which were listed before by Ridley and Turner were not recollected in this study, including the two hyper-endemics to Gunung Ledang, *Hetaeria elegans*, and *Anoectochilus burmannicus*. Checklist on the orchid species found in Gunung Ledang is provided as in Table 2.

Table 2
Checklist of Gunung Ledang orchids and the area of occurrences in Peninsular Malaysia: 122 species (from 1901-present)

No.	Species
1	*Acriopsis liliifolia* (J.Köenig) Ormerod var. *liliifolia* (Synonym: *Acriopsis javanica* Reinw. ex Blume) General Distribution: Sikkim to North West Pacific Distribution in Peninsular Malaysia: Common in lowlands throughout Lifeform: Epiphytic
2	*Aerides odorata* Lour. General Distribution: China (W. Yunnan, Guangdong) to Tropical Asia Distribution in Peninsular Malaysia: Common in lowlands throughout Lifeform: Epiphytic
3	*Agrostophyllum stipulatum* (Griff.) Schltr. subsp. s*tipulatum* General Distribution: Indo-China, Malesia to Solomon Island Distribution in Peninsular Malaysia: Johor; lowland and montane forest Lifeform: Epiphytic
4	*Anoectochilus albolineatus* C.S.P.Parish & Rchb.f. General Distribution: Indo-China Distribution in Peninsular Malaysia: Widespread; montane forest at 1000-1300 m Lifeform: Terrestrial
5	*Anoectochilus geniculatus* Ridl. General Distribution: Myanmar to West Malesia Distribution in Peninsular Malaysia: Widespread; hill and montane forest Lifeform: Terrestrial
6	*Anoectochilus burmannicus* Rolfe General Distribution: China (S. Yunnan) to Pen. Malaysia Distribution in Peninsular Malaysia: Known only from Gunung Ledang, Johor; montane forest Lifeform: Terrestrial
7	*Anoectochilus* sp. (NAJ 17) Distribution in Peninsular Malaysia: Gunung Ledang, Johor; montane forest at 1000 m Lifeform: Terrestrial
8	*Apostasia latifolia* Rolfe General Distribution: West Malesia Distribution in Peninsular Malaysia: Perak, Pahang, Melaka; hill and montane forest Lifeform: Terrestrial
9	*Apostasia nuda* R.Br. in N.Wallich General Distribution: Assam to West Malesia Distribution in Peninsular Malaysia: Widespread; lowland and hill forest to 900 m Lifeform: Terrestrial
10	*Apostasia wallichii* R.Br. in N.Wallich General Distribution: Japan (Island of Kyushu), China (South West Yunnan) to Tropical Asia and North Australia Distribution in Peninsular Malaysia: Scattered localities; lowland forest to 600 m Lifeform: Terrestrial

Table 2 (*continue*)

No.	Species
11	*Appendicula anceps* Blume General Distribution: Peninsula Thailand to Malesia Distribution in Peninsular Malaysia: Widespread and common; lowland and hill forest Lifeform: Epiphytic
12	*Appendicula cornuta* Blume General Distribution: Sikkim to China (S. Guangdong) and Malesia Distribution in Peninsular Malaysia: Common; lowland and montane forest Lifeform: Epiphytic or lithophytic
13	*Appendicula reflexa* Blume General Distribution: Taiwan, Indo-China to West Pacific Distribution in Peninsular Malaysia: Kedah, Perak, Pahang, Johor; lowlands and mountains Lifeform: Epiphytic
14	*Arundina graminifolia* (D.Don) Hochr. General Distribution: Tropical and Subtropical Asia Distribution in Peninsular Malaysia: Widespread; open sunny places in lowlands and mountains Lifeform: Terrestrial
15	*Bromheadia aporoides* Rchb.f. General Distribution: Indo-China to Borneo and Singapore Distribution in Peninsular Malaysia: South Peninsular Malaysia; lowland forest Lifeform: Epiphytic
16	*Bromheadia alticola* Ridl. General Distribution: Peninsula Thailand to Philippines (Mindanao) Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Terrestrial
17	*Bromheadia brevifolia* Ridl. General Distribution: Peninsula Thailand to West Malesia Distribution in Peninsular Malaysia: Perak, Pahang and Selangor; hill to montane forest Lifeform: Epiphytic
18	*Bromheadia finlaysoniana* (Lindl.) Miq. General Distribution: Indo-China to New Guinea Distribution in Peninsular Malaysia: Common throughout; hill to montane forest Lifeform: Terrestrial
19	*Bromheadia pungens* Ridl. General Distribution: Endemic in Peninsular Malaysia Distribution in Peninsular Malaysia: Gunung Tahan (Pahang) and Gunung Ledang (Melaka); montane forest Lifeform: Epiphytic
20	*Bromheadia rupestris* Ridl. General Distribution: Endemic in Peninsular Malaysia Distribution in Peninsular Malaysia: Gunung Jerai (Kedah), Gunung Tahan (Pahang), Pulau Tioman (Johor) and Gunung Ledang (Melaka); montane forest Lifeform: Epiphytic
21	*Bromheadia truncata* Seidenf. General Distribution: Thailand to West Malesia Distribution in Peninsular Malaysia: Widespread; lowland and montane forest Lifeform: Epiphytic
22	*Bulbophyllum clandestinum* Lindl. General Distribution: Bangladesh to West Pacific Distribution in Peninsular Malaysia: Widespread; lowland forest Lifeform: Epiphytic
23	*Bulbophyllum elevatopunctatum* J.J.Sm. General Distribution: Thailand to West Malesia Distribution in Peninsular Malaysia: Johor; lowland forest Lifeform: Epiphytic
24	*Bulbophyllum fenestratum* J.J.Sm. (Synonym: *Bulbophyllum dentiferum* Ridl.) General Distribution: Peninsula Thailand to West Malesia Distribution in Peninsular Malaysia: Kelantan, Perak, Pahang and Johor; lowland forest Lifeform: Epiphytic

Table 2 (*continue*)

No.	Species
25	*Bulbophyllum gracillimum* (Rolfe) Rolfe General Distribution: Peninsula Thailand to Southwest Pacific Distribution in Peninsular Malaysia: Common throughout; lowland forest Lifeform: Epiphytic and lithophytic
26	*Bulbophyllum purpurascens* Teijism. & Binn. General Distribution: Peninsula Thailand to West Malesia Distribution in Peninsular Malaysia: Common throughout; lowland forest Lifeform: Epiphytic and lithophytic
27	*Bulbophyllum pustulatum* Ridl. General Distribution: Peninsular Malaysia and Borneo Distribution in Peninsular Malaysia: Johor; lowland forest Lifeform: Epiphytic
28	*Bulbophyllum striatellum* Ridl. General Distribution: Peninsular Malaysia and Borneo (Sabah, Sarawak) Distribution in Peninsular Malaysia: Kelantan and Johor; lowland forest Lifeform: Epiphytic
29	*Bulbophyllum uniflorum* (Blume) Hassk. General Distribution: West and Central Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
30	*Bulbophyllum vaginatum* (Lindl.) Rchb.f. in W.G.Walpers General Distribution: Peninsula Thailand to West Malesia Distribution in Peninsular Malaysia: Common and widespread; lowland forest Lifeform: Epiphytic
31	*Calanthe angustifolia* (Blume) Lindl. General Distribution: Southeast China to West Malesia Distribution in Peninsular Malaysia: North of the Peninsula; montane forest Lifeform: Terrestrial
32	*Campanulorchis pellipes* (Rchb.f. ex Hook.f.) Y. P. Ng & P. J. Cribb General Distribution: Thailand to West Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
33	*Ceratostylis ampullacea* Kraenzl. General Distribution: Peninsula Thailand to West Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
34	*Ceratostylis gracilis* Blume General Distribution: West Malesia Distribution in Peninsular Malaysia: Pahang, Selangor; montane forest Lifeform: Epiphytic
35	*Ceratostylis subulata* Blume General Distribution: Tropical Asia to Vanuatu Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
36	*Claderia viridiflora* Hook.f. General Distribution: Peninsula Thailand to West & Central Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
37	*Cleisostoma suffusum* (Ridl.) Garay General Distribution: Peninsular Malaysia to Sumatera, North Borneo Distribution in Peninsular Malaysia: Perak, Pahang and Malacca; hill forest Lifeform: Epiphytic
38	*Coelogyne anceps* Hook.f. General Distribution: Endemic in Peninsular Malaysia Distribution in Peninsular Malaysia: Gunung Tahan (Perak, Pahang); montane forest Lifeform: Epiphytic

Table 2 (*continue*)

No.	Species
39	*Coelogyne cumingii* Lindl. General Distribution: Indo-China to West Malesia Distribution in Peninsular Malaysia: Widespread; lowland and hill forest Lifeform: Epiphytic
40	*Coelogyne kaliana* P.J.Cribb General Distribution: Endemic in Peninsular Malaysia Distribution in Peninsular Malaysia: Perak, Pahang and Selangor; montane forest Lifeform: Epiphytic
41	*Coelogyne tomentosa* Lindl. General Distribution: Peninsula Thailand to West Malesia Distribution in Peninsular Malaysia: Perak; montane forest Lifeform: Epiphytic
42	*Corybas carinatus* (J.J.Sm.) Schltr. General Distribution: West Malesia Distribution in Peninsular Malaysia: Perak, Pahang, Johor; montane forest Lifeform: Terrestrial
43	*Corymborkis veratrifolia* (Reinw.) Blume General Distribution: Tropical and Subtropical Asia to Pacific Distribution in Peninsular Malaysia: Widespread; lowland and montane forest Lifeform: Terrestrial
44	*Crepidium calophyllum* (Rchb.f.) Szlach. General Distribution: East Nepal to Hainan and Borneo Distribution in Peninsular Malaysia: Kedah, Kelantan, Pulau Pinang, Johor; hill forest Lifeform: Terrestrial
45	*Cryptostylis arachnites* (Blume) Hassk. in C.L.Blume General Distribution: Tropical & Subtropical Asia to South West Pacific Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Terrestrial
46	*Cylindrolobus nutans* (Lindl.) J.J.Wood General Distribution: Thailand to West Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
47	*Cymbidium finlaysonianum* Lindl. General Distribution: Indo-China to Malesia Distribution in Peninsular Malaysia: Most abundant in the north; lowland forest Lifeform: Epiphytic
48	*Dendrobium angustifolium* (Blume) Lindl. General Distribution: Arunachal Pradesh to China and West Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
49	*Dendrobium convexum* (Blume) Lindl. General Distribution: Indo-China to North Queensland Distribution in Peninsular Malaysia: Gunung Ulu Kali, Selangor; montane forest Lifeform: Epiphytic
50	*Dendrobium crumenatum* Sw. General Distribution: Taiwan to Tropical Asia Distribution in Peninsular Malaysia: Widespread and common; lowland forest Lifeform: Epiphytic
51	*Dendrobium derryi* Ridl. General Distribution: West Malesia Distribution in Peninsular Malaysia: Perak; montane forest Lifeform: Epiphytic
52	*Dendrobium geminatum* (Blume) Lindl. General Distribution: West Malesia Distribution in Peninsular Malaysia: Many localities on high exposed mountain ridges Lifeform: Epiphytic

Table 2 (*continue*)

No.	Species
53	*Dendrobium indivisum* (Blume) Miq. General Distribution: Bangladesh to Malesia Distribution in Peninsular Malaysia: Widespread; lowland forest Lifeform: Epiphytic
54	*Dendrobium kelsallii* Ridl. General Distribution: Endemic in Peninsular Malaysia Distribution in Peninsular Malaysia: Johor; lowland forest Lifeform: Epiphytic
55	*Dendrobium lamellatum* (Blume) Lindl. General Distribution: Java and Peninsular Malaysia Distribution in Peninsular Malaysia: Widespread but uncommon; lowland forest Lifeform: Epiphytic
56	*Dendrobium leonis* (Lindl.) Rchb.f. in W.G.Walpers General Distribution: Indo-China to West Malesia Distribution in Peninsular Malaysia: Widespread and common; lowland forest Lifeform: Epiphytic and lithophytic
57	*Dendrobium longipes* Hook.f. General Distribution: Peninsular Malaysia to West Sumatra Distribution in Peninsular Malaysia: Many localities on high exposed mountain ridges Lifeform: Epiphytic
58	*Dendrobium macropodum* Hook.f. General Distribution: West Malesia Distribution in Peninsular Malaysia: Many localities on high exposed mountain ridges Lifeform: Epiphytic
59	*Dendrobium mannii* Ridl. General Distribution: Arunachal Pradesh to Peninsular Malaysia Distribution in Peninsular Malaysia: Malacca and Johor; lowland and hill forest Lifeform: Epiphytic
60	*Dendrobium metachilinum* Rchb.f. General Distribution: Peninsula Thailand to West Malesia, Maluku (Ambon) Distribution in Peninsular Malaysia: Common in south of Peninsular Malaysia; lowland forest Lifeform: Epiphytic
61	*Dendrobium pachyglossum* E.C.Parish & Rchb.f. General Distribution: Indo-China to Peninsular Malaysia, Borneo (Sarawak) Distribution in Peninsular Malaysia: Several localities; montane forest Lifeform: Epiphytic
62	*Dendrobium uniflorum* Griff. General Distribution: Indo-China to West and Central Malesia Distribution in Peninsular Malaysia: Widespread; lowland forest Lifeform: Epiphytic
63	*Dendrobium villosulum* Wall. ex Lindl. General Distribution: Thailand, Peninsular Malaysia (P. Pinang), Borneo Distribution in Peninsular Malaysia: Widespread; lowland and montane forest Lifeform: Epiphytic
64	*Dendrochilun linearifolium* Hook.f. General Distribution: Peninsular Malaysia to Sumatra Distribution in Peninsular Malaysia: Quite widespread; montane forest Lifeform: Epiphytic
65	*Dendrochilum longifolium* Rchb.f. General Distribution: Indo-China to Papuasia Distribution in Peninsular Malaysia: Pahang southward; lowland forest Lifeform: Epiphytic
66	*Dipodium conduplicatum* J.J.Sm. General Distribution: Peninsular Malaysia to North and West Sumatra Distribution in Peninsular Malaysia: Pahang, Johor; montane forest Lifeform: Terrestrial, sometimes climbing

Table 2 (*continue*)

No.	Species
67	*Erythrodes latifolia* Blume General Distribution: West Malesia Distribution in Peninsular Malaysia: Perak, Pahang, Selangor; montane forest Lifeform: Terrestrial
68	*Erythrorchis altissima* (Blume) Blume General Distribution: Assam to Japan and Malesia Distribution in Peninsular Malaysia: Several localities; lowland forest Lifeform: Climbing holomycotroph
69	*Galeola nudifolia* Lour. General Distribution: South Hainan to Tropical Asia Distribution in Peninsular Malaysia: Widespread; lowland and hill forest Lifeform: Terrestrial holomycotroph
70	*Gastrodia javanica* (Blume) Lindl. General Distribution: Peninsula Thailand to Malesia Distribution in Peninsular Malaysia: Several localities; lowland forest Lifeform: Terrestrial holomycotroph
71	*Geodorum densiflorum* (Lam.) Schltr. General Distribution: Tropical and Subtropical Asia to West Pacific Distribution in Peninsular Malaysia: Melaka northward; open grassy places in the lowlands Lifeform: Terrestrial
72	*Goodyera rubicunda* (Blume) Lindl. General Distribution: Sikkim to Malesia to Southwest Pacific Distribution in Peninsular Malaysia: Perak, Pahang and Johor; montane forest Lifeform: Terrestrial
73	*Goodyera viridiflora* (Blume) Blume General Distribution: Tropical amd Subtropical Asia to South West Pacific Distribution in Peninsular Malaysia: Gunung Jerai (Kedah), Penang Hill, Bukit Fraser (Pahang); montane forest Lifeform: Terrestrial
74	*Grammatophyllum speciosum* Blume General Distribution: Indo-China to West Malesia Distribution in Peninsular Malaysia: Widespread; lowland and hill forest Lifeform: Epiphytic
75	*Habenaria rhodocheila* Hance General Distribution: South China to Peninsular Malaysia, Philippines Distribution in Peninsular Malaysia: Kedah, Pulau Pinang; lowland forest Lifeform: Terrestrial
76	*Hetaeria elata* Hook.f. General Distribution: Peninsular Malaysia to Philippines Distribution in Peninsular Malaysia: Pahang (Cameron Highlands); montane forest Lifeform: Terrestrial
77	*Hetaeria elegans* Ridl. (Synonym: *Hetaeria ophirensis* Ridl.) General Distribution: Endemic in Peninsular Malaysia Distribution in Peninsular Malaysia: Hyper endemic in Gunung Tunduk, Malacca; montane forest Lifeform: Terrestrial
78	*Hylophila mollis* Lindl. General Distribution: Peninsula Thailand to West Malesia and Papuasia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Terrestrial
79	*Lecanorchis malaccensis* Ridl. General Distribution: Indo-China to West Malesia Distribution in Peninsular Malaysia: Quite widespread; lowland and montane forest Lifeform: Terrestrial holomycotroph
80	*Liparis barbata* Lindl. General Distribution: Hainan to Taiwan, Tropical Asia to South West Pacific Distribution in Peninsular Malaysia: Perak, Pahang, Johor; lowland forest Lifeform: Terrestrial

Table 2 (*continue*)

No.	Species
81	*Liparis elegans* Lindl. (Synonym: *Stichorkis elegans* (Lindl.) Marg., Szlach. & Kulak) General Distribution: Nicobar Island to Hainan and South West Pacific Distribution in Peninsular Malaysia: Widespread; lowland and hills to 1000 m Lifeform: Epiphytic or lithophytic
82	*Liparis maingayi* (Hook.f.) Ridl. General Distribution: Peninsular Malaysia to West Sumatera Distribution in Peninsular Malaysia: Kedah, Pulau Pinang, Perak, Johor; hill forest Lifeform: Epiphytic
83	*Liparis viridicallus* Holttum General Distribution: West Malesia to Philippines Distribution in Peninsular Malaysia: Pahang (Fraser's Hill and Gunung Ulu Kali); montane forest Lifeform: Terrestrial or lithophytic
84	*Luisia* sp. Distribution in Peninsular Malaysia: Gunung Ledang; hill forest Lifeform: Epiphytic
85	*Macodes petola* (Blume) Lindl. General Distribution: South Japan, Peninsula Thailand to West and Central Malesia Distribution in Peninsular Malaysia: Pulau Pinang southward; damp lowland and hill forest Lifeform: Terrestrial
86	*Neuwiedia griffithii* Rchb.f. General Distribution: Vietnam, Malaya to North Sumatra Distribution in Peninsular Malaysia: Pahang, Selangor, Negeri Sembilan, Malacca, and Johor; damp lowland forest Lifeform: Terrestrial
87	*Neuwiedia veratrifolia* Blume General Distribution: Malesia to Vanuatu Distribution in Peninsular Malaysia: Pulau Pinang southward; hill and montane forest Lifeform: Terrestrial
88	*Oberonia bertoldii* King & Pantl. General Distribution: Endemic in Peninsular Malaysia Distribution in Peninsular Malaysia: Perak, Pahang, Selangor and Johor; lowland forest Lifeform: Epiphytic
89	*Paphiopedilum barbatum* (Lindl.) Pfitzer General Distribution: Peninsula Thailand to North Sumatra Distribution in Peninsular Malaysia: Widespread; open grassy or rocky places in the mountains Lifeform: Terrestrial
90	*Peristylus maingayi* (King & Pantl.) J.J.Wood & Ormerod (Synonym: *Peristylus candidus* J.J.Sm.) General Distribution: South Indo-China to North Queensland Distribution in Peninsular Malaysia: Commoner in the south of Peninsular Malaysia; montane forest Lifeform: Terrestrial
91	*Peristylus monticola* (Ridl.) Seidenf. General Distribution: Andaman Island, Malesia to New Guinea Distribution in Peninsular Malaysia: Gunung Jerai (Kedah), Gunung Ledang (Johor); montane forest Lifeform: Terrestrial
92	*Phalaenopsis deliciosa* Rchb.f. (Synonym: *Kingidium deliciosum* (Rchb.f.) H.R.Sweet) General Distribution: India to China to Malesia Distribution in Peninsular Malaysia: Widespread but not common; lowland and hill forest Lifeform: Epiphytic
93	*Phalaenopsis fuscata* Rchb.f. General Distribution: Peninsular Malaysia to Philippines Distribution in Peninsular Malaysia: Pahang and Johor; lowland forest Lifeform: Epiphytic

Table 2 (*continue*)

No.	Species
94	*Pholidota carnea* (Blume) Lindl. var. *carnea* General Distribution: Peninsular Thailand to New Guinea Distribution in Peninsular Malaysia: Perak, Pahang; montane forest Lifeform: Epiphytic
95	*Pinalia atrovinosa* (Carr) Schuit. General Distribution: Peninsular Malaysia to Borneo Distribution in Peninsular Malaysia: Pahang, Selangor and Malacca; montane forest Lifeform: Epiphytic
96	*Pinalia bractescens* (Lindl.) Kuntze General Distribution: Tropical Asia Distribution in Peninsular Malaysia: Widespread and more frequent in the south; montane forest Lifeform: Epiphytic
97	*Platanthera angustata* (Blume) Lindl. General Distribution: Hainan to West Java and Philippines Distribution in Peninsular Malaysia: Many localities; montane forest Lifeform: Terrestrial
98	*Plocoglottis javanica* Blume General Distribution: South Indo-China to West Malesia Distribution in Peninsular Malaysia: Widespread; lowland and montane forest Lifeform: Terrestrial
99	*Podochilus microphyllus* Lindl. General Distribution: Indo-China to West Malesia Distribution in Peninsular Malaysia: Widespread and common; lowland and hill forest Lifeform: terrestrial or lithophytic
100	*Pomatocalpa diffusum* Breda (Synonym: *Pomatocalpa latifolium* (Lindl.) J.J.Sm.) General Distribution: Peninsula Thailand and Malesia Distribution in Peninsular Malaysia: Widespread; lowland forest Lifeform: Epiphytic
101	*Renanthera histrionica* Rchb.f. (Synonym: *Renantherella histrionica* (Rchb.f.) Ridl.) General Distribution: Peninsula Thailand to Peninsular Malaysia Distribution in Peninsular Malaysia: Quite widespread; lowland and hill forest Lifeform: Epiphytic
102	*Rhynchostylis* sp. Distribution in Peninsular Malaysia: Gunung Ledang; hill forest Lifeform: Epiphytic
103	*Spathoglottis aurea* Lindl. General Distribution: Southeast Indo-China to West New Guinea Distribution in Peninsular Malaysia: Widespread; grassy places in the mountains at 900-1300 m Lifeform: Terrestrial
104	*Spathoglottis plicata* Blume General Distribution: Tropical and Subtropical Asia to Pacific Distribution in Peninsular Malaysia: Widespread and common; grassy places to 700 m Lifeform: Terrestrial or lithophytic
105	*Stichorkis gibbosa* (Finet) J.J.Wood General Distribution: Tropical Asia to Southwest Pacific Distribution in Peninsular Malaysia: Widespread; lowland forest Lifeform: Epiphytic
106	*Strongyleria pannea* (Lindl.) Schuit. General Distribution: East Himalaya to South China and West Malesia Distribution in Peninsular Malaysia: Common in the south; montane forest Lifeform: Epiphytic
107	*Tainia maingayi* Hook.f. General Distribution: Peninsula Thailand to West Malesia Distribution in Peninsular Malaysia: North of peninsula; montane forest Lifeform: Terrestrial

Table 2 (*continue*)

No.	Species
108	*Thecopus maingayi* (Hook.f.) Seidenf. General Distribution: South Indo-China to West Malesia Distribution in Peninsular Malaysia: Malacca; lowland forest Lifeform: Epiphytic
109	*Tainia speciosa* Blume General Distribution: Thailand to West Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Terrestrial
110	*Thrixspermum* sp. Distribution in Peninsular Malaysia: Gunung Ledang; montane forest Lifeform: Epiphytic
111	*Trichotosia ferox* Blume General Distribution: Thailand to West and South Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
112	*Trichotosia gracilis* (Hook.f.) Kraenzl. General Distribution: Indo-China to West Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
113	*Trichotosia pauciflora* Blume General Distribution: Thailand to West Malesia and Lesser Sunda Island (Bali) Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
114	*Trichotosia poculata* (Ridl.) Kraenzl. General Distribution: West Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
115	*Trichotosia velutina* (Lodd. ex Lindl.) Kraenzl. General Distribution: Arunachal Pradesh to West Malesia Distribution in Peninsular Malaysia: Widespread and common; montane forest Lifeform: Epiphytic
116	*Trichotosia vestita* (Wall. ex Lindl.) Kraenzl. General Distribution: West Malesia Distribution in Peninsular Malaysia: Widespread; montane forest Lifeform: Epiphytic
117	*Tropidia angulosa* (Lindl.) Blume General Distribution: Bhutan to South China and Lesser Sunda Island (Bali) Distribution in Peninsular Malaysia: Perak; lowland and montane forest Lifeform: Terrestrial
118	*Tropidia curculigoides* Lindl. General Distribution: East Himalaya to South China and West and Central Malesia Distribution in Peninsular Malaysia: Widespread; lowland and montane forest Lifeform: Terrestrial
119	*Vanilla griffithii* Rchb.f. General Distribution: Peninsula Thailand to West Malesia Distribution in Peninsular Malaysia: Widespread and common; lowland and montane forest Lifeform: Climber
120	*Zeuxine affinis* (Lindl.) Benth. ex Hook.f. General Distribution: Indian Subcontinent to Nansei-shoto and Peninsular Malaysia Distribution in Peninsular Malaysia: Gunung Jerai (Kedah), Penang Hill; montane forest Lifeform: Terrestrial
121	*Zeuxine gracilis* (Breda) Blume General Distribution: India to West Malesia Distribution in Peninsular Malaysia: Kedah, Penang, Pahang; montane forest Lifeform: Terrestrial

DISCUSSION

One of the interesting findings from this study is the discovery of a peculiar jewel orchid species from the genus *Anoectochilus* (*Anoectochilus* sp., NAJ17). Few individuals were observed to dwell dispersedly on the dampened ground rich in humus, in between the steep and narrow route from Gunung Tunduk towards the misty valley commencing the peak of Gunung Ledang (Figure 2). The population is very small and rare, growing among the more common dark-burgundy *Anoectochilus albolineatus*. The leaf is lime-green with interconnecting golden veins, which immediately can be easily mistaken with the velvety *Macodes petola*. However, during the visit, this unknown species of *Anoectochilus* is not in its flowering state that to proceed with taxonomic determination is a challenge. Vegetatively, the species closely resembles *Anoectochilus roxburghii* which is native to Indo-China. However, any detail on this discovery is put on hold until new information comes into light.

Figure 2. The undetermined orchid species from Gunung Ledang (*Anoectochilus* sp., NJ17). Photo by Nordin, F. A.

None of the two hyper-endemic species which have been listed previously by Turner (1995) were encountered in this study, proven by their narrow distribution and rarity. *Hetaeria elegans*, previously known as *H. ophirensis*, was discovered, and described by Ridley from Gunung Tunduk in 1908. Seidenfaden and Wood (1992) stated that *H. elegans* may be conspecific with *H. elata*, the sister species that was found to grow quite abundantly on the rich humus along the route to the bare rocky area in Gunung Tunduk at about 1200 m above sea level. Thus, more research needs to be done to resolve the taxonomic questions between the two species.

In a nutshell, Gunung Ledang was proven to be rich and diverse with its orchid flora, with Ridley's historical routes *via* Gunung Tunduk, the Lagenda Trail *via* Batu Orkid, and the peak of Gunung Ledang offer myriads of interesting discoveries. Some of the enchanting beauties are shown in Figure 3A-L. The higher peaks of Gunung Ledang were occasionally clouded in mist during the day, making them as desirable habitats for the montane orchid species. Meanwhile, the Ulu Jementah Trail *via* Jeram Tinggi worth the exploration, however fewer orchid species were counted at the lower levels of the forest. The route begins to be consistently rich with orchid species as the ascent commences Gunung Mahligai towards the peak of Gunung Ledang.

By contribution, this paper provides an updated account on the diversity of orchids in Gunung Ledang, listing 122 species of orchids, of which eight are endemic to

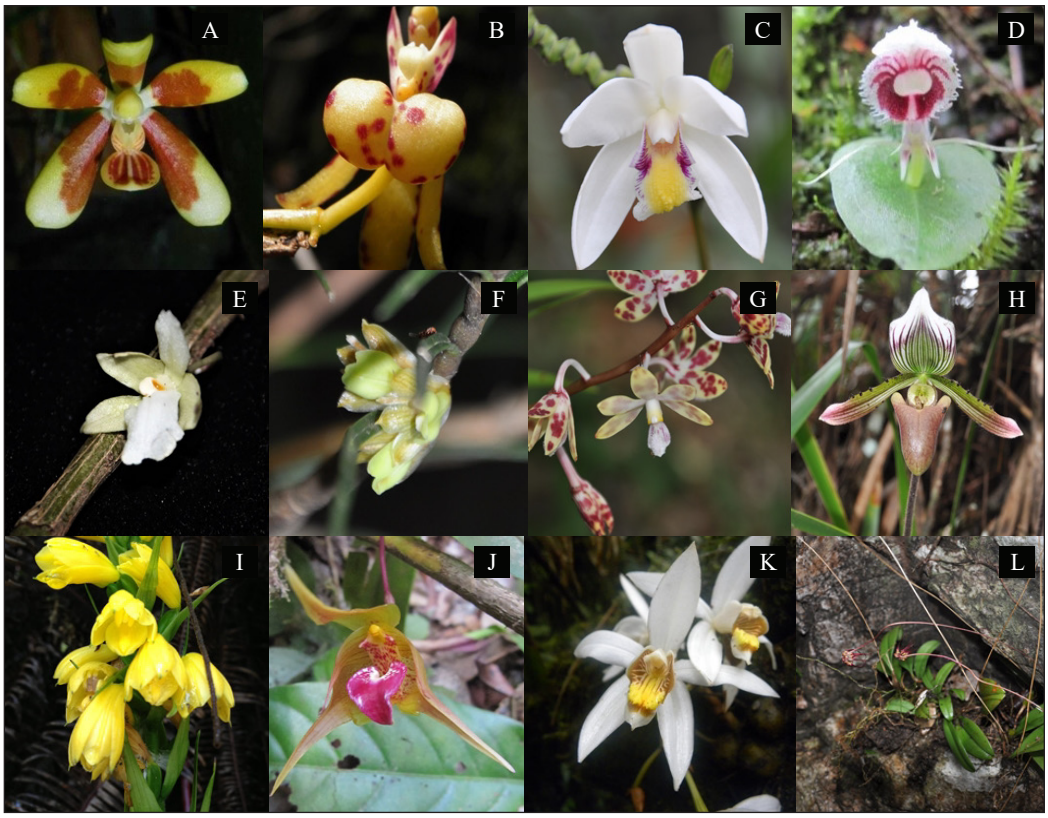

Figure 3. Myriads of orchid species from Gunung Ledang, (A) *Phalaenopsis fuscata*, (B) *Renanthera histrionica*, (C) *Bromheadia finlaysoniana*, (D) *Corybas carinatus*, (E) *Dendrobium derryi*, (F) *Dendrobium villosulum*, (G) *Dipodium conduplicatum*, (H) *Paphiopedilum barbatum*, (I) *Neuwiedia veratrifolia*, (J) *Bulbophyllum uniflorum*, (K) *Coelogyne kaliana*, and (L) *Bulbophyllum gracillimum*. Photos by Nordin, F. A.

Peninsular Malaysia, two are hyper-endemic known only from Gunung Ledang, 30 were recognised as new records, and one species needs further taxonomic clarification.

CONCLUSION

Gunung Ledang exhibits a great diversity of orchids with Ridley's historical routes were revisited and new captivating routes were explored. The 122 species in 62 genera portrayed the exceptionally rich orchid flora found on the mountain region. The decision to gazette the forests of Gunung Ledang as a national park has ensured the conservation of the rich and unique biodiversity represented in these still pristine forest areas, and especially the survival of the notable orchids as floristic heritage.

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Effect of Contact Time on the Level of Phthalates in Polyethylene Terephthalate-bottled Water from the Point of Sale

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ABSTRACT

The study aimed to evaluate the effect of contact time on the level of phthalates in polyethylene terephthalate (PET)-bottled water commercially available on the market. Different water types (drinking water, mineral water, and sparkling water) in PET bottles and mineral water in glass bottles were collected. Control (before bottling) and freshly produced (0-month) samples were collected at manufacturing sites. In contrast, samples at 6, 12, and 18 months of contact times were collected randomly from hypermarkets and supermarkets in Klang Valley, Malaysia. The samples were analyzed using LC-MS/MS with deuterated DEHP as the internal standard. DEHP, DMP, DEP, DnOP, and BBP were not detected in drinking, mineral, and sparkling water in both PET and glass bottles. However, DBP was detected within the range of 0.68 to 1.11 ng/mL for mineral water and 0.55 to 0.59 ng/mL for drinking water in PET bottles. All types of phthalates, including DBP, were not detected in the control and 0-month samples. DBP was detected at 0.59 ng/mL at 6 months of contact time and 0.55 ng/mL at 12 months

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of contact time in PET-bottled drinking water samples; the difference, however, was not significant. It appears that contact time did not significantly affect DBP levels.

Keywords: Contact time, LC-MS/MS, PET-bottled water, phthalates, point of sale

INTRODUCTION

The popularity of bottled water is rising nowadays in every part of the world with the increased usage of bottled water widely due to its convenience, inexpensive cost, and hygienic nature (Gleick, 2010). There is a rapid increase in the usage of polyethylene terephthalate (PET) bottles to package bottled water and phthalates—a type of plasticizer that is widely used in the plastic-making process (Robertson, 2013). Plasticizers are chemical additives added to polymeric products to provide durability, elasticity, and flexibility (Jia et al., 2018). Phthalates are available in free form and not chemically bound in plastic. As a result, they can potentially migrate from packaging materials into our food and beverages and cause contamination during production and storage (Gomez-Hens & Aguilar-Caballos, 2003). The compound has become a health concern due to its endocrine disrupting properties (LaFleur & Schug, 2011). Among all phthalate esters, only diethylhexyl phthalate (DEHP) is classified as a 2B substance and considered as possibly carcinogenic to humans (International Agency for Research on Cancer [IARC], 1982; Ito et al., 2019). In order to protect human health, the European Food Safety Authority established a total daily intake (TDI) for some of these pollutants; in particular, 0.05 mg/kg/bw/day for DEHP, 0.01 mg/kg/bw/day for dibutyl phthalate (DBP), and 0.5 mg/kg/bw/day for benzyl butyl phthalate (BBP) (Silano et al., 2019).

Several researchers have reported the occurrence of phthalates in bottled water. High levels of DEHP in the range of 350 µg/kg - 8.78 mg/kg were detected in bottled water in PET (Al-Saleh et al., 2011; Amiridou & Voutsa, 2011; Bosnir et al., 2007). Other researchers (Amiridou & Voutsa, 2011; Penalver et al., 2001) detected DBP in bottled water in the range of 0.1–44 ng/mL, whereas Montuori et al. (2008), as well as Al-Saleh et al. (2011), detected BBP in the range of 0.33–9.45 ng/mL. Different authors detected diethyl phthalate (DEP) in bottled water in the range of 1.536–33 ng/mL (Al-Saleh et al., 2011; Amiridou & Voutsa, 2011). Chang et al. (2015) detected di-n-octyl phthalate (DnOP) in disposable drinking water cups at 18.1 ng/mL. Fellow researchers in Malaysia, e.g., Chong et al. (2011) determined phthalates in polypropylene consumer products (such as food containers, instant noodle cups, and snack containers) and found the examined samples to contain DEHP ranging from 830 mg/kg to 1270 mg/kg. Ibrahim et al. (2014) determined phthalates in different palm oil brands, which were packed in PET bottles in the retail market in Malaysia. They detected DBP and BBP at concentrations less than 1 mg/kg. To our knowledge, there are no studies done in Malaysia on the

level of phthalates in bottled water that is commercially available on the market.

Different authors reported that the occurrence of phthalates depends strongly on the pH (carbonated vs. non-carbonated samples) (Bosnir et al., 2007; Keresztes et al., 2013; Montuori et al., 2008), storage time, storage temperature, and exposure to sunlight (Leivadara et al., 2008; Schmid et al., 2008). In addition to mineral water considered a non-carbonated drink, carbonated drinks such as soft drinks and sparkling water are also bottled using PET. Keresztes et al. (2013) reported that DBP, BBP, and DEHP were detected in non-carbonated drinks but not in carbonated mineral water samples. Besides PET, glass is also used to pack mineral water. Montuori et al. (2008) reported that the concentrations of phthalates were nearly 20 times higher in mineral water samples in PET bottles than those from glass bottles, with total levels of phthalates being at 3.52 and 0.19 ng/mL, respectively. Further, different researchers stated that aging and plastic packaging breakdown might accelerate the migration process (Amiridou & Voutsa, 2011; Rahman & Brazel, 2004). Keresztes et al. (2013) reported that DEHP could be detected after 44 days of storage at 22 °C, and its leaching was most pronounced when samples were stored for over 1200 days.

To the best of our knowledge, no study has reported the effect of contact time on the level of phthalates in PET-bottled water in Malaysia. Therefore, the present study was conducted to determine the effect of contact time on the level of phthalates, specifically DEHP, dimethyl phthalate (DMP), DEP, DnOP, DBP, and BBP in PET-bottled water that is commercially available on the market. Different types of water, i.e., drinking water, mineral water, and sparkling water in PET bottles and mineral water in glass bottles, were collected. Control (before bottling) and freshly produced (0-month) samples were collected at manufacturing sites, whereas samples at 6, 12, and 18 months of contact times were collected randomly from hypermarkets and supermarkets in Klang Valley, Malaysia. The samples were analyzed using LC-MS/MS, and deuterated DEHP was used as the internal standard.

MATERIALS AND METHODS

Characteristic of Samples

Fifty-four samples of bottled water were collected. Different types of water, i.e., drinking water, mineral water, and sparkling water in PET bottles and mineral water in glass bottles, were collected. Three different drinking water and mineral water brands in PET bottles were collected, whereas one brand of sparkling water in PET bottles and mineral water in glass bottles was collected. Samples manufactured by the following companies were collected: mineral water, drinking water, and sparkling water in PET bottles by Company A; mineral water in PET bottles by Company B and C; drinking water in PET bottles by Company D and E; and mineral water in glass bottles manufactured by Company F. In terms of size, PET-bottled water collected from Company A was in 600 mL containers, whereas PET-bottled water from other

companies was in 500 mL containers. Glass-bottled water from company F was in 1000 mL containers. Company A is located in Perak, Malaysia; Company B is located in Kedah, Malaysia; Company C, D, and E are located in Selangor, Malaysia; and company F is located in France.

In the present study, bottled water's shelf life was used to select the samples as this type of information is usually printed on the packaging. Typically, the shelf life of commercially bottled water is 24 months. However, some companies have products with a shelf life of 36 months. Control (before bottling) and freshly produced (0-month of contact time) samples were collected at manufacturing sites. Bottled water samples of other contact times, i.e., 6, 12, and 18 months, were collected randomly from hypermarkets and supermarkets in Klang Valley, Malaysia. Contact time was calculated based on the expiry date. One batch of control samples and two different batches of 0, 6, 12, and 18-months bottled samples were collected. All samples were kept sealed in their original packaging. They were stored in a refrigerator for no longer than a week before the time of analysis. Control samples were taken directly from the pipeline (before bottling) of manufacturing sites, using 500 mL glass bottles.

Phthalates Analysis

Chemicals and Stock Solutions. A high purity analytical grade of methanol was purchased from Sigma-Aldrich Chemicals (St. Louis, USA). EPA 606-M Phthalate Esters Mix (DMP, DEP, DEHP, DnOP, DBP, and BBP) with a 200 mg/mL concentration was purchased from Supelco. Deuterated phthalate (DEHP-d4), which was used as the internal standard throughout the study, was purchased from Dr. Ehrenstorfer GmbH, Germany.

A stock solution of Phthalate Esters Mix (1 mg/mL) and DEHP-d4 (100 mg/mL) was prepared by dissolving the compound in methanol. The stock solution of Phthalate Esters Mix was further diluted to prepare intermediate standard to concentrations of 50 ng/mL and 100 ng/mL with methanol. Similarly, the stock solution of DEHP-d4 was further diluted to prepare a working standard to concentrations of 1 mg/mL with methanol. All stock solutions and intermediate standards were stored in a refrigerator at 4 °C for a maximum of 1 year.

Sample Preparation. The procedure described by Schreiber et al. (2011) was followed. A 1 mL water sample was accurately transferred to a 10 mL volumetric flask and made up with methanol. A 100 ml of 100 ng/mL internal standard (DEHP-d4) was added to the mixture and shaken well. The mixture was then allowed to stand for 10 minutes before being transferred into a vial for LC-MS-MS analysis.

LC-MS/MS Analysis. The detection of phthalates in bottled water was performed on PerkinElmer Flexar UHPLC AS system (PerkinElmer, Waltham, Massachusetts, US) coupled with 3200 QTRAP® Linear Ion Trap Quadrupole LC-MS/MS operated in

multiple reaction monitoring (MRM) mode (AB Sciex, Framingham, Massachusetts, USA). The standards contained 0.5, 1, 5, 10, 20, 50 ng/mL and DEHP-d4 at 10 ng/mL. Twenty (20) µL was used as the injection volume.

Separation of phthalates was achieved under gradient conditions using Phenomenex Synergi Fusion-RP C18 (100 mm x 2.0 mm x 2 µm) column and fast gradient water + 0.1% formic acid and acetonitrile with 0.1% formic acid at mobile phase with a flow rate of 400 µL/min. The following MS/MS transitions were monitored: (i) m/z 313>205 for BBP, (ii) 391>261 for DnOP, (iii) 195>163 for DMP, (iv) 391>279 for DEHP, (v) 223>177 for DEP, (vi) 279>205 for DBP, and (vii) 395>171 for DEHP-d4.

Quantification. The transitions of m/z 313>205 for BBP, 391>261 for DnOP, 195>163 for DMP, 391>279 for DEHP, 223>177 for DEP, 279>205 for DBP, and 395>171 for DEHP-d4 were used for quantification. Furthermore, m/z 313>149 for BBP, 391>149 for DnOP, 195>133 for DMP, 391>167 for DEHP, 223>149 for DEP, 279>149 for DBP, and 395>153 for DEHP-d4 were used as confirmation of peak identity. A calibration graph was constructed by plotting phthalates' peak areas relative to the internal standard against the corresponding ratios of analyte amounts. Phthalate levels in samples were calculated from the calibration slope and intercept value. The calibration curve for each phthalate esters was linear; DMP (r=0.9958), DEP (r-0.9984), DEHP (r-0.9950), DnOP (r=0.9959), DBP (r=0.9980), and BBP (r=0.9961). The detection limit (LOD) was 0.5 ng/mL, and recoveries were in the range of 70–120%.

Statistical Analysis

All data obtained in this study were analyzed using SPSS Version 21.0 (SPSS Inc., Chicago, IL). One-way ANOVA with Tukey's test was used to determine the differences in phthalates levels among different contact times. The *p*-value of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

A total of 54 bottled water samples were analyzed for phthalates at different contact times: 0, 6, 12, and 18 months with samples before bottling as the control. Table 1 shows that DEHP, DMP, DEP, DnOP, and BBP were not detected in different brands of drinking, mineral and sparkling water in PET bottles, and mineral water in glass bottles at different contact times. The findings are in agreement with Ceretti et al. (2010), who reported that DEHP was not detected in PET-bottled mineral water. Similarly, Guart et al. (2011) did not detect DEHP, DMP, or BBP in any bottled water, whether in PET or glass bottles. However, numerous authors reported high levels of phthalates in bottled water, especially DEHP (Al-Saleh et al., 2011; Amiridou & Voutsa, 2011; Bosnir et al., 2007), DEP (Al-Saleh et al., 2011; Amiridou & Voutsa, 2011), and BBP (Al-Saleh et al., 2011; Montuori et al., 2008). Besides, Table 1 shows that DBP was detected at 0.59 ng/

mL at 6 months of contact time and 0.55 ng/mL at 12 months of contact time in PET-bottled drinking water samples (brand E). The difference in the means, however, was not significant. It appears that contact time did not significantly affect DBP levels. The finding seems to be in contrast with Keresztes et al. (2013), who reported that the leaching of DEHP (i.e., another type of phthalate) was the most pronounced when mineral water in PET was stored for over 1200 days at 22 °C. Keresztes et al. (2013) performed an experimental study in which researchers introduced intervention and studied the effects. However, the present study is observational in which researchers observed the effect of contact time as a risk factor without trying to influence how bottled waters are handled. To obtain insight into the actual level of phthalates in bottled water as consumed by general consumers, it is necessary to collect samples at the point of sale, in which samples were already subjected to different handling practices by different stakeholders along the supply chain. Although the drinking water samples at each contact time were collected from the same manufacturer (brand E), they are of different samples as they were gathered from different batches of production and already subjected to different handling practices along the supply chain. It might explain why the present study did not observe any significant effect of contact time on DBP levels.

In addition, DBP was detected in the range of 0.68 to 1.11 ng/mL in different brands of mineral water and 0.55 to 0.59 ng/mL in different brands of drinking water in PET bottles. The findings agree with Serodio and Noqueira (2006) who reported that DBP was the most abundant phthalate in bottled mineral water from a Portuguese spring, with a level of 0.35 ng/mL. Moreover, all types of phthalates, including DBP, were not detected in the freshly produced (0-month of contact time) and the before bottling (control) samples. It might be possible that the sources of water collected in the present study, i.e., from the states of Perak, Kedah, and Selangor, are free from phthalate contamination. Possibly, DBP that was detected at 6, 12, and 18 months was due to contamination during the bottling or handling processes. Different authors suggested that the primary sources of phthalate contamination in bottled water could be from the PVC tubes used in municipal distribution (Hahladakis et al., 2018; Sulentic et al., 2018).

DBP was detected at 0.68 ng/mL (brand A) and 1.11 ng/mL (brand C) for PET-bottled mineral water at 6 months of contact time. Similarly, at 0.58 ng/mL (brand A) and 0.59 ng/mL (brand E) for PET-bottled drinking water. The difference, however, was not significant (Table 1). Furthermore, DBP was not detected in PET bottles' sparkling water (brand A) at different contact times. It appears that the type of bottled water does not significantly affect DBP levels. Sparkling water is an example of carbonated drinks, whereas mineral and drinking water that are also in PET bottles are examples of non-carbonated drinks. Different authors compared phthalate levels

Table 1

Level of phthalates in bottled water (ng/mL)

Phthalates	Type of packaging	Type of water	Brand	Contact time				
				Before bottling (control)	0 month	6 months	12 months	18 months
DMP[1]	PET[7]	Mineral water	A	<LOD[8]	<LOD	<LOD	<LOD	<LOD
			B	<LOD	<LOD	<LOD	<LOD	<LOD
			C	<LOD	<LOD	<LOD	<LOD	<LOD
		Drinking water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			D	<LOD	<LOD	<LOD	<LOD	<LOD
			E	<LOD	<LOD	<LOD	<LOD	<LOD
		Sparkling water	A	ND[9]	ND	ND	<LOD	<LOD
	Glass	Mineral water	F	ND	ND	ND	<LOD	ND
DEP[2]	PET	Mineral water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			B	<LOD	<LOD	<LOD	<LOD	<LOD
			C	<LOD	<LOD	<LOD	<LOD	<LOD
		Drinking water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			D	<LOD	<LOD	<LOD	<LOD	<LOD
			E	<LOD	<LOD	<LOD	<LOD	<LOD
		Sparkling water	A	ND	ND	ND	<LOD	<LOD
	Glass	Mineral water	F	ND	ND	ND	<LOD	ND

Table 1 *(Continued)*

Phthalates	Type of packaging	Type of water	Brand	Contact time				
				Before bottling (control)	0 month	6 months	12 months	18 months
DEHP[3]	PET	Mineral water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			B	<LOD	<LOD	<LOD	<LOD	<LOD
			C	<LOD	<LOD	<LOD	<LOD	<LOD
		Drinking water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			D	<LOD	<LOD	<LOD	<LOD	<LOD
			E	<LOD	<LOD	<LOD	<LOD	<LOD
		Sparkling water	A	ND	ND	ND	<LOD	<LOD
	Glass	Mineral water	F	ND	ND	ND	<LOD	ND
DnOP[4]	PET	Mineral water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			B	<LOD	<LOD	<LOD	<LOD	<LOD
			C	<LOD	<LOD	<LOD	<LOD	<LOD
		Drinking water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			D	<LOD	<LOD	<LOD	<LOD	<LOD
			E	<LOD	<LOD	<LOD	<LOD	<LOD
		Sparkling water	A	ND	ND	ND	<LOD	<LOD
	Glass	Mineral water	F	ND	ND	ND	<LOD	ND

Table 1 *(Continued)*

Phthalates	Type of packaging	Type of water	Brand	Contact time				
				Before bottling (control)	0 month	6 months	12 months	18 months
DBP[5]	PET	Mineral water	A	<LOD	<LOD	0.68±0.96[10,A]	<LOD	ND
			B	<LOD	<LOD	<LOD	<LOD	1.01±0.09
			C	<LOD	<LOD	1.11±0.59[A]	<LOD	<LOD
		Drinking water	A	<LOD	<LOD	0.58±0.81[A]	<LOD	<LOD
			D	<LOD	<LOD	<LOD	<LOD	<LOD
			E	<LOD	<LOD	0.59±0.82[aA]	0.55±0.77[a]	<LOD
		Sparkling water	A	ND	ND	ND	<LOD	<LOD
	Glass	Mineral water	F	ND	ND	ND	<LOD	ND
BBP[6]	PET	Mineral water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			B	<LOD	<LOD	<LOD	<LOD	<LOD
			C	<LOD	<LOD	<LOD	<LOD	<LOD
		Drinking water	A	<LOD	<LOD	<LOD	<LOD	<LOD
			D	<LOD	<LOD	<LOD	<LOD	<LOD
			E	<LOD	<LOD	<LOD	<LOD	<LOD
		Sparkling water	A	ND	ND	ND	<LOD	<LOD
	Glass	Mineral water	F	ND	ND	ND	<LOD	ND

Note. [1]Dimethyl phthalate; [2]Diethyl phthalate; [3]Di-2-ethylhexyl phthalate; [4]Di-n-octyl phthalate; [5]Dibutyl phthalate; [6]Benzylbutyl phthalate; [7]Polyethylene terephthalate; [8]LOD = 0.5 ppb; [9]ND= no data; [10]Mean value based on N=2; [a-b] Values within the same row with different lowercase letters are significantly different ($p \leq 0.05$); [A-B] Values within the same column with different uppercase letters are significantly different ($p \leq 0.05$)

for non-carbonated and carbonated drinks and found that the rate of DBP migration was higher for non-carbonated than carbonated drinks (Bosnir et al., 2007; Keresztes et al., 2013). The present study, however, did see such an observation. The finding may be because the present study is observational, and samples for each contact time were collected from different production batches and already subjected to different handling practices, as explained above.

All types of phthalates, including DBP were not detected in the mineral water in glass bottles (brand F) in contrast to the above-mentioned DBP levels in mineral and drinking water in PET bottles. The findings are consistent with the findings of Montuori et al. (2008), who reported that the concentrations of phthalates were nearly 20 times higher, i.e., determined at 3.52 ng/mL in mineral water samples in PET bottles compared to glass bottles (0.19 ng/mL). Besides, the United States Environmental Protection Agency regulates DEHP through the National Primary Drinking Water Regulations under the Safe Drinking Water Act. The maximum contaminant levels of DEHP in drinking water has been set at 6.0 ng/mL (United States Environmental Protection Agency [US EPA], 1991). Since the levels of all phthalates in this study were below the maximum limit set for DEHP, which is 6 ng/mL, the bottled water that is commercially available on the market is considered as safe from phthalate contamination. However, it should be noted that there are no international guidelines for DBP or other phthalates in drinking water except for DEHP (Al-Saleh et al., 2011).

CONCLUSION

The present study showed that DEHP, DMP, DEP, DnOP, and BBP were not detected in the bottled water samples collected at different contact times. DBP was detected in the PET-bottled drinking water samples (brand E) with 6 months and 12 months of contact times, although the difference was not significant. It appears that contact time does not significantly affect DBP levels. All types of phthalates, including DBP were not detected in the following samples: control (before bottling), freshly produced (0-month), sparkling water in PET-bottles, and mineral water in glass bottles. Phthalates in all samples did not exceed the maximum established limit of DEHP (<6 ng/mL). This study will serve as a reference for future researchers in determining the dietary exposure of phthalates from bottled water.

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Wildlife Crossings at Felda Aring - Tasik Kenyir Road, Malaysia

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ABSTRACT

The Felda Aring - Tasik Kenyir Road was identified as one of the most threatening roads to wildlife in Malaysia. The present study was conducted to assess the road crossing activities involving the medium- to large-mammal species due to the problem stated. The objectives of this study were to (1) predict the suitability of the road and its surroundings as the roaming areas for the Asian elephant (*Elephas maximus*, n = 104) and Malayan tapir (*Tapirus indicus*, n = 66), (2) identify the mammalian species inhabiting the forest beside the road, (3) compare the forest's common species [photographic capture rate index (PCRI) > 10/ detection probability (P) ≥ 0.05] with the ones utilising the road crossing structures; the viaducts and the bridges, and (4) determine the most impacted species from traffic collisions. The road and its surroundings were classified as moderately suitable to the elephant and tapir (suitability values = 0.4 - 0.8). A total of 16 mammal species were recorded at the forest edges, in which the wild pig (*Sus scrofa*) (PCRI = 118.96, P = 0.3719 ± 0.027), barking deer (*Muntiacus muntjak*)

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(PCRI = 68.89, P = 0.2219 ± 0.0232), sun bear (*Helarctos malayanus*) (PCRI = 11.13, P = 0.0507 ± 0.0159), tapir (PCRI = 11.13, P = 0.0469 ± 0.0118), elephant (PCRI = 10.7, P = 0.0787 ± 0.0195), and Malayan porcupine (*Hystrix brachyura*) (PCRI = 10.7, P = 0.103 ± 0.0252) were the common species utilising the crossing structures. In contrast, the Asian palm civet (*Paradoxurus hermaphroditus*) and leopard cat (*Prionailurus bengalensis*) were the most frequently hit species on the road [$F(7,398) = 28.53$, $p < 0.0005$]. The present study found that large-mammal species were utilising the crossing structures at a higher frequency, whereas more medium-mammal species were involved in traffic collisions.

Keywords: Camera trapping, fragmentation, GIS mapping, roadkill, viaducts

INTRODUCTION

Southeast Asia is home to many endangered megafauna species, including the Asian elephant (*Elephas maximus*), Malayan tiger (*Panthera tigris*), and Malayan tapir (*Tapirus indicus*). These species' population are declining primarily due to habitat loss (Chwalibog et al., 2018; Fernando & Pastorini, 2011; García et al., 2012). In most Southeast Asian countries, the forest area is decreasing with rapid road development. For example, between 2009 - 2016, the evergreen forest and deciduous forest in Thailand were reduced by 8% and 11%, respectively (Trisurat et al., 2019). In 2011 – 2012, Indonesia, the most forested Southeast Asian country, experienced the world's highest annual rate of forest loss (2.2 million hectares) and expansion of road network by 42% (Alamgir et al., 2019).

Malaysia has undergone rapid infrastructure growth, with 122% growth in road length in a decade. In 2016, the total road length in Peninsular Malaysia was 177,569 km (CEIC, 2016). Malaysia has progressed and gained tremendous success in economic growth and productivity due to its rapid infrastructure development. However, rapid urbanisation has had a significant impact on the surrounding environment.

Major road expansion, particularly within natural habitats, obstructs wildlife's movement and ability to utilise resources. Roads reduce landscape permeability and connectivity by acting as barriers to animal movement through traffic collisions and habitat fragmentation (Ahmad Zafir & Magintan, 2016). The number of wildlife-vehicle collisions (WVC) will most likely increase with rapid road development (Alamgir et al., 2018). In Peninsular Malaysia, there were 350 mammalian individuals killed between 2006 - 2009, 605 between 2010 - 2014, and 535 between 2012 - 2017 due to WVC (Jamhuri et al., 2020; Kasmuri et al., 2020; Sukami, 2016). From this number, the wild pig (*Sus scrofa*), leopard cat (*Prionailurus bengalensis*), long-tailed macaque (*Macaca fascicularis*), Asian palm civet (*Paradoxurus hermaphroditus*), and Malayan tapir were among the most frequently hit species (Jamhuri et al., 2020). A total of 15 individuals of Malayan tapir

were killed between 2006 - 2010 (Magintan et al., 2012), while another 68 were killed between 2012 - 2017 (Kasmuri et al., 2020). A study also found that some of the Peninsular Malaysia roads acted as a solid barrier to elephant movements, with 80% reduction in permeability (Wadey et al., 2018).

Several initiatives from the Malaysian government have been apprehended to mitigate wildlife issues due to road expansion. The Central Forest Spine (CFS) is an important national land-use master plan for maintaining wildlife habitat connectivity across major forest blocks in Peninsular Malaysia (Department of Town and Country Planning, 2009). A study has identified 16 main roads in Southeast Asia that are threatening mammal habitats in which three of them are located in primary linkage (PL) 1 (Tanum Forest Reserve (FR) - Sungai Yu FR), PL 2 (Temengor FR - Royal Belum State Park), and PL 7 (Taman Negara National Park-Tembat FR) of CFS 1 (Clements et al., 2014). The implementation of the CFS plan involved the construction of several viaducts under those three roads (Kasmuri et al., 2020) to reduce road implications and facilitating the wildlife movement, particularly the large-mammal species such as the sun bear (*Helarctos malayanus*), gaur (*Bos gaurus*), Asian elephant, Malayan tiger, and Malayan tapir (Magintan, 2012).

The PL 7 is an important wildlife corridor aimed to maintain the connection between the Taman Negara National Park with Tembat FR despite being bisected by the road. This connection is crucial as both areas are identified as wildlife hotspots in Peninsular Malaysia (Ratnayeke et al., 2018). While the road's impact has been somewhat mitigated by constructing three viaducts along the Felda Aring - Tasik Kenyir Road, WVC involving large-mammal were still reported (Clements et al., 2012b). This manuscript highlights the road crossing activities, particularly involving the medium- to large-mammal species using the crossing structures (CS) under the road; the viaducts and the bridges. The road and its surrounding's suitability as the roaming areas for the Asian elephant and Malayan tapir were accessed as both species were recognised among the most impacted large-mammal species from road development. Besides, the viaducts were built primarily to assist the movement of both species.

To assess the crossing activities at the Felda Aring - Tasik Kenyir Road, the objectives of the present study were to (1) predict the suitability of the road and its surrounding as the roaming areas for the Asian elephant and Malayan tapir, (2) identify the mammalian species inhabiting the forest beside the road, (3) compare the forest's common species with the ones utilising the road CS; the viaducts and the bridges, and (4) determine the most impacted mammal species from WVC. In achieving the objectives, camera traps were used to record the mammal species inhabiting the forest edges and utilising the CS. Maximum entropy algorithm (MaxEnt) was applied to predict the road's suitability as a wildlife roaming area, and roadkill events involving

medium- to large-mammal species along the road were recorded continuously during the sampling period.

MATERIALS AND METHODS

Study Area

The present study was conducted along the Felda Aring - Tasik Kenyir Road, situated in Tembat FR (Figure 1), the largest FR in Terengganu, Malaysia (1346.92 km^2). Located in eastern Peninsular Malaysia, Tembat FR (5°20' to 4°50'N and 102°20' to 102° 60'E) serves as an important connection between the Titiwangsa-Bintang-Nakawan Ranges Forest Complex and the Taman Negara National Park-Eastern Ranges Forest Complex. The highest peak of Tembat FR is the Tembat Mountain, reaching 965 m from sea level. Located near the equator, this rainforest experiences a hot and humid climate throughout the year, with an average temperature ranging from 28 °C to 30 °C during the daytime and relatively cooler at night. The total rainfall at Tembat FR in 2018 was 2320 mm, and April was the driest month (55 mm), while December was the wettest (552 mm).

All the study sites, the forest edges, viaducts, and standard bridges are located along the Felda Aring - Tasik Kenyir Road (Figure 1). The forest edges were the most extensive study site, which acts as the control site representing the natural habitat for mammal species inhabiting the study area. The forest edges data was used as the baseline data to predict the mammal species using the CS to cross the road. The sampling stations in the forest edges were located at (mean ± SD) 166.50 ± 174 m from the road. Chen and Koprowski (2016) found that some animals did not avoid entering roadside areas, and the probability of crossing random line transects in the forest edges was not affected by distance to roads. According to a study conducted in Peninsular Malaysia, the closest distance between a roadkill location and forest is 4.2 ± 0.3 km (Jamhuri et al., 2020). With reference to these studies, the mammal species recorded at the forest edges have the potential to cross the road.

The viaducts and bridges are the concrete CS built under the road (Figure 2). The Malaysia Public Works Department created the viaducts in 2008 to assist wildlife movement between forest patches. Shrubs and grasses dominated both CS types with fewer existing trees of (mean ± SD) height = 8.35 ± 5.80 m and diameter at breast height (dbh) = 5.40 ± 5.24 cm. In contrast, the vegetation in the forest edges was dominated mainly by understories of bekak (*Aglaia malaccensis*) [Ivi(%) = 6.75], membuluh (*Pellacalyx saccardianus*) [Ivi(%) = 5.46], and huru (*Beilschmiedia madang*) [Ivi(%) = 5.05].

The three viaducts (located between Felda Aring, Kelantan and Kenyir Reservoir, Terengganu) are situated inside the PL 7 (Figure 1) serve as important linkages between the Taman Negara National Park and Tembat - Lebir FRs. Construction of Viaduct 1 (V1: length = 245 m, width = 11.90 m, height = 14.50 m), Viaduct 2 (V2: length = 140 m, width = 12.95 m, height = 13.93 m), and Viaduct 3 (V3: length = 245 m, width = 10.30 m, height = 15.40 m) were

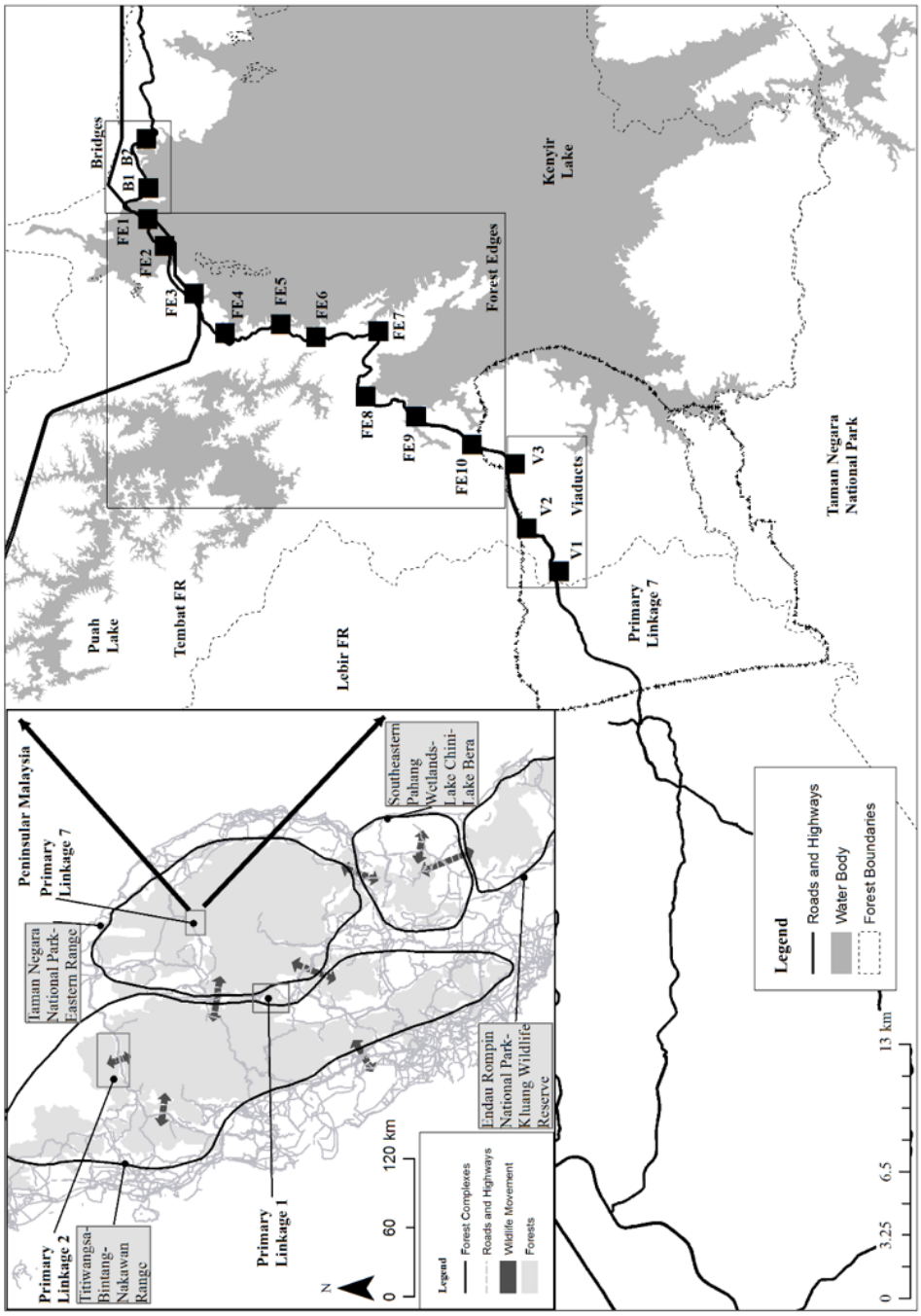

Figure 1. The camera trap stations along the Felda Aring - Tasik Kenyir Road, the road which bisects the primary linkage 7 (PL 7) of Central Forest Spine

(a)

(b)

Figure 2. (a) The viaduct, which was built to assist the wildlife movement; (b) The bridge, which was built to connect the road

Photo credit: Mr. Mohd. Faiz bin Mohd. Yusoff

completed in 2008 (Magintan, 2012) at the cost of RM30 million (Kawanishi, 2014) to mitigate the impact of road development on wildlife movement inside the PL 7 area. The viaducts were built at ±10 m higher than the average height of adult Asian elephants (240 cm - 300 cm) (Sukumar, 2006) to provide a spacious crossing area to the large-mammal. The animal trails are accessible on both sides of the viaducts.

The CS was built across existing rivers, namely Sg. Kembur at V1, Sg. Kelampai at V2, and Sg. Purun at V3. Pastures of 20,200 m^2 were planted at V2, and artificial salt licks were introduced at each viaduct by the Department of Wildlife and National Park Peninsular Malaysia (DWNP) (Bakri et al., 2019). In 2018, ten local fruit tree species were planted at the viaducts as part of the present study with the Jabatan Perhutanan Negeri Terengganu's supervision to rehabilitate the area. 'Tampoi' (*Baccaurea kunstleri*), 'santol' (*Sandoricum koetjape*), Malay apple (*Syzygium malaccense*), gandaria (*Bouea macrophylla*), 'jering' (*Archidendron jiringa*), 'asam gelugur' (*Garcinia atroviridis*), 'kerdas' *(Archidendron bubalinum*), bitter bean (*Parkia speciosa*), 'kembang semangkuk' (*Scaphium longiflorum*), and wild almond (*Irvingia malayana*) were the species planted.

Seven other bridges were constructed outside of the PL 7 along the road to meet engineering purposes. However, these bridges were not specifically designed for wildlife crossings (Department of Town and Country Planning, 2009). Most of the bridges were built to provide passage across the Tasik Kenyir and its tributaries. The bridges were built at the two-way road with guardrails installed on both sides with no divider built in the middle. The forest edges and hillside terraces sandwich the road (Figure 2). Two bridges; Bridge 1 (B1: length = 140.39 m, width = 9.30 m, height = 17.70 m) and Bridge 2 (B2: length = 166.06 m, width = 9.36 m, height = 17.37 m) were selected as the study sites. The selected bridges are the farthest bridges from the nearest village; Kampung Basung, Hulu Terengganu. Bridges were selected based on the level of human disturbance to avoid vandalism of the camera traps. Bridges without wildlife passage and dominated by water bodies were not selected as the study sites.

Data Gathering and Analysis

Prediction of Roaming Areas for *Elephas maximus* and *Tapirus indicus* Near Felda Aring - Tasik Kenyir Road. The distribution of *E. maximus* (n = 104) and *T. indicus* (n = 66) at the road and its surroundings were predicted using the occurrence data (n) collected from the year 2003 - 2008 (Figure 3). The occurrence data was based on sightings, footprints, faecal matter, and feeding signs of both species, collected by the DWNP rangers during their routine patrol in the forests. Factors including slope, elevation, land use types, soil types, and distance to rivers and roads were used to predict the distribution (Table 1). All the variables were converted into a raster format. The correlation between each

parameter was tested using the Pearson correlation in which the variables with a correlation coefficient $r > \pm 0.8$ were excluded from the analysis. This step was taken to reduce errors in the contribution of interrelated variables in the prediction (Yi et al., 2016). Subsequently, the selected variables were converted to ASCII format and analysed by MaxEnt software version 3.3.3a with the default setting (convergence threshold = 10^{-5}, maximum iterations = 500, regularisation parameter of 1.0). MaxEnt consists of an algorithm that estimates the probability of distribution and produces the most uniform information in the targeted area (Phillips et al., 2006). In this study, the PL 7 and the Felda Aring - Tasik Kenyir Road suitability as wildlife roaming areas were assessed based on the predicted distributions of both *E. maximus* and *T. indicus*.

Table 1

The variables used for assessing suitable roaming areas for Tapirus indicus *and* Elephas maximus *using the maximum entropy algorithm*

Variables	Unit/Format	Data source	Type
Land Use 1 = Non-agriculture 2 = Forest 3 = Road and utility 4 = Mine 5 = Others 6 = Urban 7 = Agriculture 8 = Water body	–	JUPEM	Categorical
Distance to river	Kilometre (km)	JUPEM	Continuous
Distance to roads and urban	Kilometre (km)	JUPEM	Continuous
Altitude	Metre (m)	SRTM	Continuous
Gradient (derive from altitude)	Degree (°)	SRTM	Continuous
Lithology 1 = Intrinsic acid rocks, mud, and clay 2 = lime stones and alluvium	–	JMG	Categorical

Note. JUPEM = Department of Survey and Mapping Malaysia, SRTM = Shuttle Radar Topography Mission, JMG = Mineral and Geoscience Department Malaysia

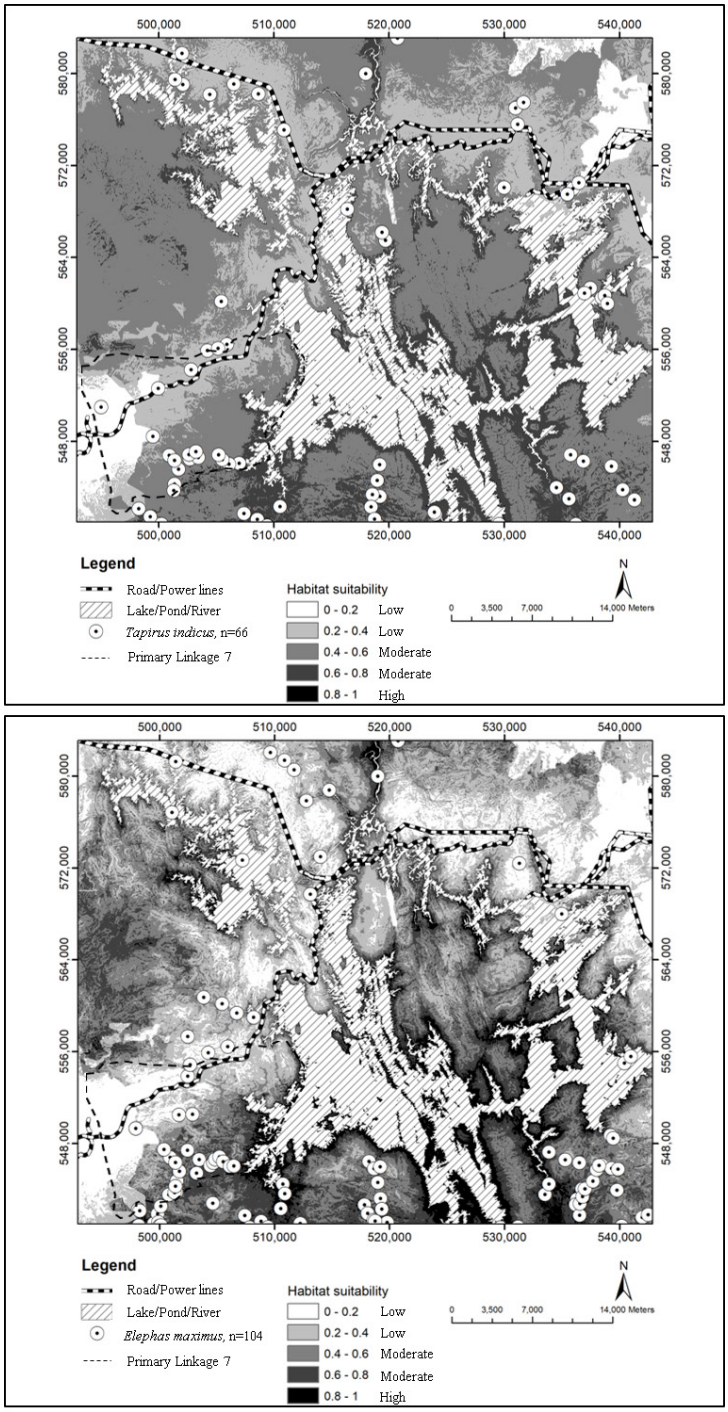

Figure 3. Prediction of the Felda Aring - Tasik Kenyir Road suitability as roaming area for *Tapirus indicus* (n = 66) and *Elephas maximus* (n = 104) based on the GIS: maximum entropy algorithm. The predictive maps were reclassified into five classes with the logistic threshold of 0.2

Camera Trapping. Digital camera traps (16 MP, 20 m infrared night vision, IP65 water resistance, ARTITAN brand) were used to record the presence of wildlife in the study area. The camera traps with 0.6 s trigger speed were powered by four AA-sized alkaline batteries and equipped with an eight GB SD card. A total of 33 camera traps were positioned in all study sites covering a whole area of 75.01 km^2 in which 20 camera traps were placed at ten stations in the forest edges, nine camera traps at the viaducts, and four camera traps at the bridges.

Camera trapping layouts used for data collection in the forest edges included systematic placement and random allocation following the road as a line transect. In contrast, deliberately-biased placement was applied at both CS types (Meek et al., 2014). The starting point for camera trapping was initiated at a random position in the forest edges while the other stations were located at 2.36 ± 1.2 km intervals. Two camera traps were placed at each station with a distance of 210.79 ± 185 m from each other. Random allocation was added to the systematic placement if needed due to habitat and geographical constraints. The deliberately-biased placement was used at the CS targeting the crossing area located under the road as the focal point. The number of camera trap replicates at each CS depended on the CS's length, column structures distance, and river width, including two to three camera traps at approximately 64 ± 15 m intervals.

The camera traps were operated for eight months from December 2017 to July 2018. All wildlife images captured due to motion and heat triggers were defined as events. In contrast, consecutive images of different individuals of different species, consecutive images of individuals of the same species taken more than 60 minutes apart (Meek et al., 2014), and non-consecutive images of individuals of the same species were defined as independent events (N) (O'Brien et al., 2003). The camera traps were set to capture three photos in one trigger with a 60 s delay between each trigger. All camera traps were mounted on trees at least 0.3 m (Meek et al., 2014) above the ground, perpendicular and approximately 3 m to the animal trails in the forest edges. At the CS, the camera traps were mounted on a 1 m steel pole at least 0.3 m above the ground. Habitat clearing was performed at all study sites to avoid false triggers by leaves and clear the view. No bait was used to prevent recapturing images of the same individuals. The changing of batteries, retrieval of images, and camera trap replacement due to theft and damage were performed after at least one month of operation.

All captured images were identified to species level with the aid of Francis (2019) and sorted through the digiKam 7.2.0 photo management application. To avoid sequences of photos of a particular individual, a period of 60 minutes was used to differentiate the individual mammal photographs. The camtrapR package (Niedballa et al., 2016) in R-3.5.0 software (R Core Team, 2019) implements a temporal independence filter between images of the same species at the same station (argument minDeltaTime

= 60 minutes). By setting to 60 minutes, the number of independent events of each species at each station was produced from images taken at least 60 minutes after the same species/individual's last record at the same station (Niedballa et al., 2016). All functions for downstream analysis depend on the number of independent events produced.

The most common mammal species in the forest edges that crossed both CS types were identified. Each species' independent events from each camera trap (N) were used to calculate the photographic capture rate index (PCRI) in each study site using the equation: PCRI = N*1000/∑trap nights (Table 2). Each species' PCRI was assigned to three rank abundance categories, i.e., < 10 = rare species, 10 - 100 = common species, and > 100 = abundant species (Bartholomew, 2017). PCRI was used to reduce bias in the frequency of detection when sample sizes were unequal for each study site.

Furthermore, the relative abundance index (RAI) for each sampling station was calculated (Table 3), and a map of RAI versus species richness was developed to quantify all 15 stations' effectiveness as wildlife crossings independently (Figure 4). Species richness was defined as the total number of mammal species detected at each station over the entire camera trap duration, and the relative abundance was calculated using the equation: RAI = 100*(N/Σtrap nights). RAI was used to reduce bias among stations by standardising each station's independent events into 100 days of sampling efforts (Clements, 2011).

The camtrapR package also computes detection/non-detection data (1, 0) for use in occupancy analysis. One camera trap week (7 days) was compressed to represent each survey. PRESENCE software (Hines, 2006) was used to obtain the occupancy estimates (Ψ) and detection probabilities (P) of each species at each study site (Table 2).

Roadkill. The WVC involving medium- to large-mammal species along the Felda Aring - Tasik Kenyir Road was recorded during the eight-month sampling period. Data were collected by four observers in a moving vehicle [Toyota Hilux Double Cab 2.4G (MT) 4 x 4] at a speed of 50 km/h using a GPS (Garmin GPSMAP 64s) and a digital camera (Olympus TG-870). Surveys were made during early hours every three days, checking both sides of the road. Whenever a road-killed animal was detected, the carcass was identified to the lowest possible taxonomy, pictures were taken, and the geographic coordinate position was recorded with 3 m accuracies. By referring to Santos et al. (2011), three days were selected as the survey's interval in the present study since medium- to large-species had the longest persistence time on the road, which is three to seven days. A graph of roadkill number versus species was plotted to determine the most frequently hit species on the road during the sampling period, and multivariate analysis was conducted to support the findings.

Table 2

*The independent events (N) of each mammal species recorded in each study site at 60 minutes intervals. The photographic capture rate index (PCRI) was used to identify the common mammal species in the forest edges using the equation: N*1000/ ∑ trap nights. Occupancy (Ψ) and detection probability (P) was calculated from detection/non-detection data grouped into camera trap weeks*

Sampling stations			Forest edges (FE)				Viaducts (V)				Bridges (B)			
Sampling efforts (days)			2337				1838				516			
Order	Species	Common name	N	PCRI	Ψ	P	N	PCRI	Ψ	P	N	PCRI	Ψ	P
Artiodactyla	*Capricornis sumatraensis*	Sumatran serow	3	1.28	1 ± 0	0.0094 ± 0.0054	0	—	—	—	0	—	—	—
	*Muntiacus muntjak**	Barking deer	161	68.89	1 ± 0	0.2219 ± 0.0232	228	124.05	1 ± 0	0.7742 ± 0.0434	7	13.57	1 ± 0	0.1714 ± 0.0637
	*Sus scrofa**	Wild pig	278	118.96	1 ± 0	0.3719 ± 0.027	50	27.2	1 ± 0	0.3118 ± 0.048	1	1.94	1 ± 0	0.0256 ± 0.0253
	Tragulus sp*	Mouse-deer	25	10.7	0.64 ± 0.1683	0.083 ± 0.0215	0	—	—	—	0	—	—	—
Carnivora	*Canis lupus familiaris*	Domestic dog	2	0.86	1 ± 0	0.0062 ± 0.0044	0	—	—	—	0	—	—	—
	Catopuma temminckii	Asian golden cat	4	1.71	0.6435 ± 0.5405	0.0194 ± 0.0184	0	—	—	—	0	—	—	—
	Paguma larvata	Masked palm civet	0	—	—	—	1	0.54	1 ± 0	0.0104 ± 0.0104	0	—	—	—
	Panthera pardus	Leopard	5	2.14	1 ± 0	0.0156 ± 0.0069	0	—	—	—	0	—	—	—
	*Pardofelis marmorata**	Marbled cat	4	1.71	0.2467 ± 0.1679	0.0507 ± 0.0318	0	—	—	—	0	—	—	—
	Prionailurus bengalensis	Leoprad cat	0	—	—	—	10	5.44	0.6721 ± 0.2745	0.144 ± 0.0454	0	—	—	—
	Martes flavigula	Yellow-throated marten	2	0.86	1 ± 0	0.0062 ± 0.0044	0	—	—	—	0	—	—	—

Table 2 *(Continued)*

Sampling stations			Forest edges (FE)				Viaducts (V)				Bridges (B)			
Sampling efforts (days)			2337				1838				516			
Order	Species	Common name	N	PCRI	Ψ	P	N	PCRI	Ψ	P	N	PCRI	Ψ	P
	*Helarctos malayanus**	Sun bear	26	11.13	0.987 ± 0.1994	0.0507 ± 0.0159	0	—	—	—	1	1.94	1 ± 0	0.0256 ± 0.0253
	Paradoxurus hermaphroditus	Asian palm civet	1	0.43	1 ± 0	0.0031 ± 0.0031	3	1.63	1 ± 0	0.0323 ± 0.0183	0	—	—	—
	Viverra tangalunga	Malayan civet	2	0.86	1 ± 0	0.0062 ± 0.0044	1	0.54	1 ± 0	0.0108 ± 0.0107	0	—	—	—
	Viverra zibetha	Large Indian civet	0	—	—	—	5	2.72	0.822 ± 0.3937	0.0523 ± 0.0328	0	—	—	—
Perissodactyla	*Tapirus indicus**	Malayan tapir	26	11.13	1 ± 0	0.0469 ± 0.0118	76	41.35	1 ± 0	0.4194 ± 0.0512	15	29.07	1 ± 0	0.2564 ± 0.0699
Primates	*Macaca nemestrina**	Southern pig-tailed macaque	12	5.13	0.316 ± 0.1537	0.089 ± 0.031	0	—	—	—	0	—	—	—
	Macaca fascicularis	Long-tailed macaque	0	—	—	—	0	—	—	—	1	1.94	1 ± 0	0.0208 ± 0.0206
Proboscidea	*Elephas maximus**	Asian elephant	25	10.7	0.7549 ± 0.1613	0.0787 ± 0.0195	13	7.07	1 ± 0	0.0968 ± 0.0307	0	—	—	—
Rodentia	*Hystrix brachyura**	Malayan porcupine	25	10.7	0.5159 ± 0.1638	0.103 ± 0.0252	11	5.98	0.3333 ± 0.2722	0.3226 ± 0.084	0	—	—	—

Note. Species* = Common mammal species recorded in the forest edges with PCRI > 10/ P ≥ 0.05; N = Independent events from Camera traping; PCRI = Photographic capture rate index; Ψ = Occupancy estimates; P = Detection probabilities

Table 3

The total number of species and independent events (N) in each study site. The relative abundance index (RAI) was calculated to standardise the sampling efforts of each sampling station into 100 days using the equation: 100(N/Σtrap nights), where N = independent events at 60 minutes intervals*

Sampling stations	Total number of species	N	RAI
FE1	7	57	2.44
FE2	4	18	0.77
FE3	8	46	1.97
FE4	9	44	1.88
FE5	10	171	7.32
FE6	7	76	3.25
FE7	6	18	0.77
FE8	8	66	2.82
FE9	6	59	2.52
FE10	12	46	1.97
V1	7	98	5.33
V2	8	160	8.71
V3	6	140	7.62
B1	3	16	3.1
B2	4	9	1.74

Note. N = Independent events from Camera trapping; RAI = Relative abundance index

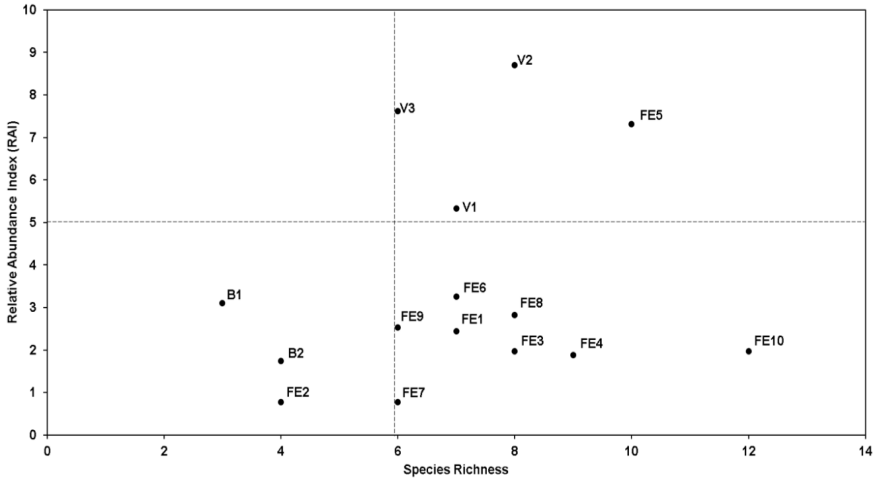

Figure 4. Each camera trap station was plotted based on the relative abundance index (RAI) and species richness. RAI was calculated using the equation: 100*(N/Σtrap nights), where N = independent events at 60 minutes intervals. Each camera trap station is representing a wildlife crossing area

RESULTS

Prediction of Roaming Areas for *Elephas maximus* and *Tapirus indicus* Near Felda Aring - Tasik Kenyir Road

The present study generated a useful MaxEnt prediction with an area under the curve (AUC) > 0.7 in which values close to 1 refer to high prediction model accuracy (Yang et al., 2013; Yi et al., 2016). Based on the selected variables collected before the enrichment activities, the model categorised the study area into five classes with suitability values ranging from 0, the lowest suitability value to 1, the highest suitability value (Figure 3). The maps in Figure 3 show that majority of the study area was moderately suitable as roaming areas for both *T. indicus* (n = 66) and *E. maximus* (n = 104) with suitability values of 0.4 - 0.8. However, this suitability was disconnected at the Felda Aring - Tasik Kenyir Road, indicated with suitability values between 0 and 0.4. Most of the PL 7 area, including the viaducts, was recognised as less to moderately suitable as a wildlife crossing area with suitability values ranging from 0 to 0.8. The viaducts, however, are sandwiched by moderately suitable habitats with suitability values between 0.4 and 0.8.

Camera Trapping

The camera trap sampling efforts include a total of 4,691 trap nights, which exclude the days when the cameras were not functioning due to technical problems, getting stolen, and being damaged. These sampling efforts yield a total of 3,282 events of 33 wildlife species comprised of six birds, two reptiles, and 25 mammal species, including five small and 20 medium- to large-mammal species. Out of all recorded medium- to large-mammal species, 16 were recorded at the forest edges while ten were recorded at the viaducts, and five were recorded at the bridges (Table 2). Based on the PCRI, the most common large-mammal species in the forest edges (PCRI > 10) were the *S. scrofa* (PCRI = 118.96), barking deer (*M. muntjak*) (PCRI = 68.89), *H. malayanus* (PCRI = 11.13), *T. indicus* (PCRI = 11.13), and *E. maximus* (PCRI = 10.7) while the mouse-deer (*Tragulus* sp.) (PCRI = 10.7) and Malayan porcupine (*H. brachyura*) (PCRI = 10.7) were the most common medium-mammal in the forest edges.

The probability of detecting a species in the forest edges during a survey (P) was higher (≥ 0.05) for the *S. scrofa* (P = 0.3719), *M. muntjak* (P = 0.2219), *E. maximus* (P = 0.0787), *H. malayanus* (P = 0.0507), and *T. indicus* (P = 0.0469). Besides, the probability of detecting a *Tragulus* sp. (P = 0.083), *H. brachyura* (P = 0.103), marbled cat (*Pardofelis marmorata*) (P = 0.0507), and pig-tailed macaque (*Macaca nemestrina*) (P = 0.089) was higher compared to other medium-mammal. Four of the listed common large-mammal species were recorded at both CS types, except for *H. malayanus* at the viaducts and *E. maximus* at the bridges while *H. brachyura* was the only common medium-mammal species recorded at the viaducts. Based on this analysis, the usage of both CS types by the common large-mammal was nearly equal to that observed in the forest edges.

The RAI versus species richness map was generated to compare each camera trap station's effectiveness as a wildlife crossing (Figure 4). The map classifies stations with high species richness and high RAI (indicates a higher frequency of mammals' crossings) at its top-right quadrant and *vice versa*. All viaducts were categorised as effective wildlife crossings since all of them were plotted on the top-right quadrant. In contrast, the bridges were classified as the least effective wildlife crossings since both were plotted on the bottom-left quadrant. The map also indicates V2 as the most effective crossing compared to the other viaducts.

Roadkill

A total of five medium- to large-mammal species were killed along the Felda Aring - Tasik Kenyir Road during the sampling period due to WVC (Figure 5). From those species, *P. hermaphroditus* and *P. bengalensis* recorded the highest number of cases (n = 4), followed by *S. scrofa* (n = 2), silvery langur (*Trachypithecus cristatus*), and spectacled langur (*Trachypithecus obscurus*) (n = 1), in which *S. scrofa* and *P. hermaphroditus* were the only species recorded at the viaducts. There was a statistically significant difference in road crossing activities (roadkill versus viaduct) based on species from multivariate analysis, $F(7,398) = 28.53$, $p < 0.0005$. Post hoc comparison using the Tukey HSD test indicated that the mean score for road crossing activities was statistically significantly different between *P. hermaphroditus* and *P. bengalensis* with other species ($p < 0.005$), which means that both species are at risk to be involved in WVC compared to other

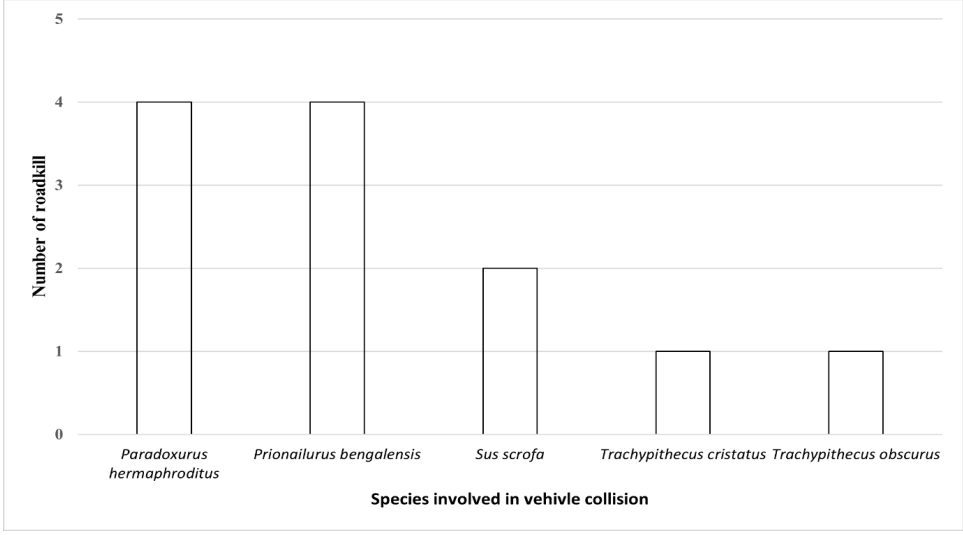

Figure 5. Medium- to large-mammal species involved in wildlife-vehicle collisions along the Felda Aring - Tasik Kenyir Road during the sampling period

mammal species, that are utilising the viaducts. There was no roadkill recorded for the common medium- to large-mammal species utilising the CS, i.e., *E. maximus*, *M. muntjak*, *T. indicus*, *H. malayanus*, and *H. brachyura*, except for *S. scrofa*. Besides, no roadkill recorded for other medium-mammal species utilising the viaducts, i.e., the masked palm civet (*Paguma larvata*), Malayan civet (*Viverra tangalunga*), and large Indian civet (*Viverra zibetha*).

DISCUSSION

Prediction of Roaming Areas for *Elephas maximus* and *Tapirus indicus* Near Felda Aring - Tasik Kenyir Road

Prediction of wildlife distribution is one of the analyses that must be performed to identify the forest's connectivity zones to mitigate the road impacts toward wildlife (Poor et al., 2012). MaxEnt algorithm is the widely used landscape analysis that produces good predictions of species distribution (Poor et al., 2012) compared to other species distribution models (SDM) such as analytic hierarchy process (AHP), genetic algorithm for rule-set production (GARP), BIOCLIM, and DOMAIN. MaxEnt is based on a machine learning response designed to make predictions from incomplete input information (Baldwin, 2009), such as previous distribution data with the selected set of environmental, climatic, and spatial variables of the input (Abidin et al., 2019). MaxEnt also has been used to predict suitable habitats for wildlife species (Kabir et al., 2017; Mohd Taher et al., 2017), which is essential in predicting the habitat connectivity zones (Mohd Taher et al., 2017). A study found that 10 out of 12 published works implementing SDM in Malaysia used MaxEnt as their preferred modelling method (Rahman et al., 2019).

The *H. malayanus* was listed as one of the common large-mammal species in the study area. However, this species was recorded only once at the CS, suggesting road avoidance behaviour. This finding is supported by Nazeri et al. (2012), which found that the sun bear has a strong preference for dense forests and avoids open areas and roads based on the SDM.

The low habitat suitability values at the viaducts were anticipated because roads were recognised as barriers to most of the wildlife species by SDM (Angelieri et al., 2016; Radnezhad et al., 2015). This finding is similar to a study conducted in Taman Negara National Park which found that the suitable habitat for *Tragulus* sp. was not connected due to the presence of road between forest blocks (Mohd Taher et al., 2018). However, the moderately suitable habitats, including the Taman Negara National Park, Tembat FR, and Lebir FR, which surround the viaducts, are predicted to enhance viaducts' usage and the PL 7 as well. The strategic location of viaducts facilitates mammals' movement across the road and directly increases viaducts' usage. Hence, the present study supports the selection of viaducts' location and the PL 7 area as an ecological linkage since this area is located between the important wildlife roaming habitats (Magintan et al., 2015).

The road's suitability value as roaming areas for *T. indicus* was higher than *E. maximus* indicates that *T. indicus* has a higher chance of crossing the road. From 2006-2010, 142 individuals of Malayan tapir were displaced from its natural habitats in Peninsular Malaysia. From this number, 27 individuals have died due to injuries and WVC (Magintan et al., 2012). The high PCRI and P-value at viaducts and no roadkill recorded in the study area indicate viaducts' success in attracting utilisation by the Malayan tapir.

Camera Trapping

A field survey of medium- to large-mammal species by camera trapping is required to support the predictive model developed by MaxEnt. MaxEnt algorithm works with single species (Baldwin, 2009) and provides a useful predictive model for a species. However, many other important wildlife species inhabit the study area, which also needs to be surveyed. Hence, camera trapping is required to collect simultaneous multiple species data in the study area (Burgar et al., 2018). The results from both analyses can be compared to determine the effectiveness of mitigation measures taken at the low suitability areas predicted by undertaking the field survey after completing the mitigation plans, such as the habitat enrichments.

The present study revealed a slightly decreasing pattern of mammal species diversity than the previous studies conducted in the same area. Asian wild dog (*Cuon alpinus*), bearcat (*Arctictis binturong*), and Malayan tiger, which were recorded by previous studies (Clements, 2013; Nurul-Adyla et al., 2016), were not recorded in the present study. Moreover, Nurul-Adyla et al., (2016) did not record the presence of Sambar deer (*Rusa unicolor*) and clouded leopard (*Neofelis nebulosa*), which were previously recorded by Clements (2013). However, the abundance of *S. scrofa* and *M. muntjak* in the present study was similar to that reported in the previous studies. The decreasing pattern of mammal diversity in the study area is most probably related to the clear-felling of natural forests to develop the hydroelectric dam in Tembat FR and Petuang FR in 2013 (Clements, 2013). About 43.41% increase of logged-over forest was detected in the same year (Magintan et al., 2017). In 2016, the water body's size increased by 96.60% from its initial size due to the dam's impoundment. A total of 244 mammals were rescued during the impoundment; however, no conflict or rescue of large mammals recorded (Nur-Syuhada et al., 2016). Large mammals were expected to leave the habitat, as reported by Magintan et al. (2020).

Similar to the present study, camera trap studies in Jerangau FR and Taman Negara Kelantan and Terengganu managed to record the presence of arboreal species; *M. fascicularis* and *M. nemestrina* (Abd Gulam Azad, 2006; Jambari et al., 2015). However, other species such as *Prebytis* sp. and *Trachypithecus* sp. could not be recorded by the camera traps due to their strictly arboreal behaviour. Because of the

restriction, road crossing activities involving these species could not be assessed.

Sampling effort was the highest at the forest edges, and the chosen area was the largest to maximise capture rate (Tobler et al., 2008) to determine the most common large-mammal in the study area. The presence of most of the common large-mammal at the CS indicates the success of both CS types in attracting the common large-mammal to utilise a narrow and limited wildlife crossing. This interpretation was made with reference to Srbek-Araujo and Chiarello (2013), which found that the mammal communities sampled in the forest were supposed to differ significantly from those sampled on the roads. Furthermore, the comparable results among the viaducts and bridges also revealed the potential of standard bridges to indirectly facilitate road crossing at a reduced cost. The present study found the similarity in the number of species recorded at the forest edges and the viaducts, suggesting some similarity between enriched limited space located under the road and the unlimited space above the road.

The presence of the common large-mammal species was used as the subject for comparison due to the higher requirements for this group to move between habitat patches because of their larger home range sizes (Abd-Gani, 2010; Abidin et al., 2019; Bahar et al., 2018). Due to that reason, *E. maximus* was expected to be recorded at all study sites. Nevertheless, this species was not recorded at any bridge. Although the bridges can become a safer crossing option for wildlife, this CS type was considered unfavourable to the elephants. An underpass is considered to be safer when it can reduce WVC (Magintan, 2012) and functional when it can reduce mortality and increase movement, meet the animals' biological requirements, facilitate dispersal and recolonisation, and assist redistribution of populations in response to disturbance (Clevenger & Huijser, 2011).

In some parts of this county, major roads increase the possibility of direct mortality due to WVC (Abd Gulam Azad, 2006). A study found that the Asian elephant is attracted to the roadsides (Wong et al., 2018a). A total of 750-road crossing events were recorded during the study period (Wadey et al., 2018). Due to their requirements to cross the road, many roadkill events involving this species were reported (Jamhuri et al., 2020; Kasmuri et al., 2020; Sukami, 2016; Timbuong, 2019; Wong et al., 2018a). These roadkill events can be reduced if the wildlife is attracted to cross the road via a bridge that is free from traffic.

Factors including the lack of habitat enrichment activity, essential sources, and other physical factors might cause elephants' avoidance of the bridges. The frequent presence of elephants on the East-West Highway (Wadey et al., 2018; Wong et al., 2018b) and the placement of warning signages for elephants on the road (Timbuong, 2019) show a high tendency for this species to not using the CS. On the other hand, the viaducts were explicitly designed to connect the elephants' main landscapes (Saaban et al., 2011).

The high species richness and RAI at viaducts justify that area's fitness as a suitable wildlife crossing area (Clements, 2011). This fitness, which was previously absent based on SDM, resulted from the government's financial support to mitigate road development's impact on wildlife movement inside the PL 7 area (Department of Wildlife and National Park [DWNP], 2013).

Both *H. brachyura* and *Tragulus* sp. were the common medium-mammal species in the forest edges based on the PCRI and P values. However, *Tragulus* sp. was neither recorded at any CS nor involved in WVC. According to Mohd Taher et al. (2018), *Tragulus* sp.'s habitat is more dependent on the river's presence but away from the urban area, such as roads. On the other hand, *H. brachyura* was frequently involved in WVC (Jamhuri et al., 2020; Kasmuri et al., 2020). The presence of this species at viaducts suggests viaducts' success in attracting the utilisation by *H. brachyura*.

The camera traps positioned at the viaducts captured a higher frequency of human images than other study locations. Researchers and local villagers were among the recorded humans at the viaducts. Since most of the mammalian species in the forest edges were recorded at the viaducts, the present study supports that most mammalian species can adapt to human presence. Furthermore, no significant differences in mammals' activity patterns recorded from the camera traps in the National Park between the open and closed tourist seasons suggest that human presence has limited effects on wild mammals' behaviour (Ota et al., 2019).

Habitat Enrichment

Several habitat enrichment activities such as the deployment of artificial salt licks (Bakri et al., 2019; Magintan et al., 2015), the establishment of pastures (Bakri et al., 2019; DWNP, 2013), and the planting of local fruit trees (Shu-Aswad Shuaib, 2017) taken near the viaducts were among the factors that improve the usage of the viaduct and facilitates the large-mammals' movement across the road. These strategies are essential in upgrading the less suitable habitats (based on the MaxEnt analysis) to an effective wildlife crossing (based on the field survey data). The essential resources provided at the PL 7, including additional food, minerals, and water, have successfully attracted the herbivores to cross the road safely, although roads are known as one of the leading causes of wildlife avoidance (Fahrig & Rytwinski, 2009).

The PCRI and P values of *M. muntjak* and *T. indicus* at the viaducts were higher than other species suggesting viaducts' success in attracting a very high frequency of herbivore species. The strategic location of viaducts that connect the two main forest blocks (Saaban et al., 2011) also provides more suitable areas for wildlife crossings than other stations. The higher preference for viaducts was also aided by the rivers as a water source and dense bushes, which are one of the preferred herbivores' habitat features (Farida et al., 2006).

Roadkill

The success of enrichment activities taken at the viaducts in attracting wildlife to cross-over was observed. This finding also shows that the risk for wildlife not using the viaducts as a crossing option is low, except for certain medium-sized species such as *P. hermaphroditus* and *P. bengalensis*. Nonetheless, both species are commonly involved in traffic collisions (Kasmuri et al., 2020); as such, *P. bengalensis* was usually hit in the agricultural area, especially near oil palm and rubber plantations (Laton et al., 2017). The researchers also recorded a higher frequency of *P. hermaphroditus* on the roads compared to the forests (Wilting et al., 2010). This species uses roads as an excretory site, which is vital as a medium for communication (Nakabayashi et al., 2014). The presence of *P. hermaphroditus* on the roads also related to the abundance of plant food such as 'sesendok' (*Endospermum diadenum*) and figs (*Ficus* spp.) along the roadside (Nakashima et al., 2010).

The viaducts have successfully become the preferred crossing option for the large-sized herbivores based on their abundance at the viaducts and no roadkill recorded throughout the study period. Kasmuri et al. (2020) reported that among the most frequently hit species in Peninsular Malaysia was the *T. indicus*. Although no roadkill involving *T. indicus* was recorded in the present study, this species is at risk of being hit at any roads in Peninsular Malaysia since the forest in this country contains 69% of its predicted suitable habitats (Clements et al., 2012a). On the other hand, there was no roadkill recorded for the barking deer in Peninsular Malaysia between 2012-2017 (Kasmuri et al., 2020). This result suggests the road avoidance behaviour adopted by this species, instead choosing to utilise the viaducts in the PL 7.

The present study found that the medium-mammal species was frequently hit at the road compared to the large-mammal. This finding suggests the enrichment of the CS to attract higher medium-mammal utilisation, particularly *P. hermaphroditus* and *P. bengalensis*. Fruit trees such as papaya (*Carica papaya*) and jackfruit (*Artocarpus heterophyllus*) can be planted to attract *P. hermaphroditus* and the prey of *P. bengalensis* (Jothish, 2011).

CONCLUSION

The present study had successfully recorded the utilisation of the Felda Aring - Tasik Kenyir Road as mammals' crossings. It was found that the road's surrounding was moderately suitable as roaming areas for both *Elephas maximus* and *Tapirus indicus*, and this suitability serves as a benchmark for assessing the roaming areas for other herbivorous species. Most of the common large-mammal species inhabiting the forest edges were recorded utilising the CS, and the viaducts were categorised as the most effective wildlife crossing along the road. However, the medium-mammal species were frequently hit on the road. The present study found that the essential resources provided near the viaducts are crucial to increasing the viaducts' utilisation, encouraging safer road crossings, and

facilitating the wildlife movement, primarily the herbivorous across forest patches. The present study suggests a follow-up study in the future to assess the viaducts usage when the trees planted at the viaducts start to bear fruits. It is predicted that the viaducts' utilisation will become more critical in the future when the road usage is increased and the surrounding habitat is reduced.

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In silico Characterization of the Structure of Genes and Proteins related to β-carotene Degradation in *Musa acuminata* 'DH-Pahang' and *Musa balbisiana* 'Pisang Klutuk Wulung'

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ABSTRACT

β-carotene is an important nutritional content in banana. However, its lifetime depends on the enzymes controlling its conversion into strigolactone. To understand the involved enzymes' activity, which are β-carotene isomerase (D27), carotenoid cleavage dioxygenase 7 (CCD7), and CCD8, would be the key to manipulate the rate of β-carotene degradation. In this research, the structure of genes and proteins of the D27, CCD7, and CCD8 from *Musa acuminata* were characterized. 'DH-Pahang' and *Musa balbisiana* 'Pisang Klutuk Wulung' (PKW). The corresponding sequence of genes from both species were aligned to determine similarity and intron/exon positions. Domains and motifs in the sequences of putative proteins of D27, CCD7, and CCD8 were also identified. It was found that *D27*, *CCD7*, and *CCD8* genes in DH-Pahang and PKW comprise of various nucleotide sequence length, putative proteins, and numbers and length of exons and introns. However, the putative proteins possess the same domains: DUF4033 (domain of unknown function) in D27 and RPE65 (retinal pigment epithelium) in CCD7 and CCD8. Phylogenetic trees showed that D27, CCD7, and CCD8 proteins from DH-Pahang and PKW are conserved and clustered in

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the same clades with the same proteins of monocot plants. Hence, the results could be useful for future research in optimizing β-carotene content in banana.

Keywords: A genome, B genome, β-carotene, *CCD*, *D27*

INTRODUCTION

Banana (*Musa* sp.) is a fruiting herb that is one of the most exported and consumed plants globally. Banana has many cultivars (Calberto et al., 2015). There are four genome types of banana, i.e. A, B, S, and T genomes. Banana cultivars mainly descended from *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). The Cavendish cultivar of *M. acuminata* (AAA genome) dominates 90% of banana export value (Davey et al., 2013). The amount of β-carotene is 0.2 mg/100 grams (dry weight/d.w.) of fruit flesh, similar to *M. balbisiana* (0.19 mg/100 grams (d.w.) of fruit flesh) (Mathew & Muhammed, 2015). The β-carotene content in a plant is affected by three aspects: biosynthesis, degradation or conversion to another molecule, and storage in a plant. However, in other banana plants such as *Musa troglodytarum* (T genome) that contains a higher amount of β-carotene (1.5 mg/100 grams (d.w.) of fruit flesh) (Englberger et al., 2006), no significant differences were found when Buah (2015) compared the structure and expression of β-carotene biosynthesis genes between *M. troglodytarum* and *M. acuminata*. Therefore, we focus on the degradation aspect.

One of the carotenoid's degradation pathways is the conversion from carotenoid to strigolactone; a phytohormone that plays an essential role in inducing mutualistic symbiosis between fungi and plant roots in the form of mycorrhiza (Alder et al., 2012). Strigolactone plays a vital role in managing plant response to stress (Mishra et al., 2017). The pathway of β-carotene conversion to strigolactone involves three enzymes that are expressed from the genes *D27* (*DWARF27*) and *carotenoid cleavage dioxygenase 7* and *8* (*CCD7* and *CCD8*) (Alder et al., 2012).

Whole-genome sequencing of A genome for the cultivar DH-Pahang and B genome for the cultivar 'Pisang Klutuk Wulung' (PKW) has been conducted by D'Hont et al. (2012) and Davey et al. (2013). The genes in the whole genome sequence (WGS) of DH-Pahang have been predicted and available online at GenBank. However, the genes in the WGS of PKW have not been wholly annotated.

In this research, the structures of the genes *D27*, *CCD7*, and *CCD8* in *M. acuminata* cultivar DH-Pahang and *M. balbisiana* cultivar PKW were characterized *in silico*. In this work, the functions of the genes were also analyzed by identifying putative protein domains and motifs *in silico* and constructing phylogenetic trees for putative protein sequences of D27, CCD7, and CCD8. From this study, researchers were able to understand more about the genes, and hope that it could be beneficial for future biofortification efforts in banana.

MATERIALS AND METHODS

WGS of DH-Pahang and PKW Sequence Data Retrieval

D27, *CCD7*, and *CCD8* genes in the WGS of DH-Pahang were annotated *in silico* and available in NCBI GenBank (Clark et al., 2016). The gene sequences were predicted *in silico* from the annotation of genes in Eukaryotes (in this case, the *M. acuminata*). Meanwhile, the genetic sequences of PKW were retrieved by BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) from the genes of DH-Pahang to the WGS of PKW, by using the application blast2seq in NCBI with the algorithm optimization megablast so that the resulting sequences are highly similar to the genes from DH-Pahang. Sequences with the highest max score were chosen and then predicted to be the nucleotide sequences of *D27*, *CCD7*, and *CCD8* in PKW. For reference, a search for those genes in banana genomes based on the same genes in *Arabidopsis thaliana* as a model organism was also done.

Prediction of Gene Structure, Motifs, and Domains of D27, CCD7, and CCD8 Putative Proteins of DH-Pahang and PKW

Prospective nucleotide sequences were then predicted and annotated to obtain gene structure, putative amino acid sequences, and predicted protein motifs and domains. To predict gene structure and putative amino acid sequences, the program FGENESH (Solovyev et al., 2006) was used. FGENESH predicts exon-intron structure in a gene and predicts putative amino acid sequences. Exon and intron mapping were also validated by sim4 (Florea et al., 1998), by mapping mRNA putative sequences into respective genes' sequences. Exon and intron structures of PKW's genes were then compared to DH-Pahang's genes. Then, protein motifs and domains were predicted using the NCBI Conserved Domain Search (Marchler-Bauer et al., 2017) and MEME-Suite (Bailey & Elkan, 1994). Motif identification was done using the motif search in InterProScan (Jones et al., 2014).

Comparison of Gene Structure and Putative Proteins of D27, CCD7, and CCD8 between DH-Pahang and PKW

Predicted nucleotide structure of *D27*, *CCD7*, and *CCD8* were compared for each gene, between DH-Pahang and PKW. Gene structures compared were nucleotide sequence length, number of exons and introns, and position for each exon and intron. Predicted protein structures were compared between DH-Pahang and PKW; the comparisons were motif and domain positions in the protein and length of amino acid sequences that made up those motifs and domains. Aside from that, pairwise alignment between the respective genes and proteins between the two cultivars was determined to determine similarity and identity percentage between the genes/proteins. The pairwise alignment was done by using EMBOSS-Needle (Madeira et al., 2019).

Construction of Phylogenetic Trees of D27, CCD7, and CCD8 Genes and Putative Proteins between DH-Pahang, PKW, and Other Plant Species

Phylogenetic analyses were done to determine the similarity and genetic relation of D27, CCD7, and CCD8 putative proteins in A and B genome bananas and other monocot plants with similar genes (paralogous to banana). The phylogenetic tree was made based on the putative amino acid sequence of D27, CCD7, and CCD8 in DH-Pahang, PKW, and other plants. Amino acid sequences of those proteins in different plants were obtained by using BLAST towards the GenBank database. Aside from paralogous sequences, nucleotide and amino acid sequences from *A. thaliana* were also retrieved to serve as an outgroup in the phylogenetic tree.

After sequences were retrieved, they were aligned globally with T-Coffee v.11, UNIX-based (Notredame et al., 2000), and trimmed with BioEdit v.7.2.6 (Hall, 1999). Phylogenetic trees were constructed with MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Parameters used were invgamma for rates (among-site rate variation), nGen (number of generations) variable from 10,000 to 20,000 trees, with sampling frequency for every 100 trees. Phylogenetic trees constructed were then visualized with TreeView v.1.6.6 (Page, 1996).

RESULTS AND DISCUSSION

D27, *CCD7*, and *CCD8* Genes in DH-Pahang and PKW Based on Reference Nucleotide Sequences in GenBank and Genetic Sequences of *Arabidopsis thaliana*

Table 1 shows a summary of the genes (detailed in Figure 1) DH-Pahang and PKW. Figures 2-4 show the exon-intron structure (part-a), the protein domain structure (part-b), and the putative protein motive structure (part-c) in genes *D27*, *CCD7* as well as *CCD8* in DH-Pahang and PKW.

Table 1
*Genes involved in the conversion of β-carotene into strigolactone in DH-Pahang (*Musa acuminata*) and PKW (*Musa balbisiana*)*

No.	Gene name	Species	GeneID	Seq. length (bp)	Located in chromosome no.	Number of exons	% Similarity
1	*D27*	*Musa acuminata*	103986218	3,532	1	7	41
		Musa balbisiana		2,602		5	
		Musa acuminata	103976367	2,891	2	6	97.2
		Musa balbisiana		2,897		5	
2	*CCD7*	*Musa acuminata*	103972006	3,768	11	7	95.4
		Musa balbisiana		3,723		5	
3	*CCD8*	*Musa acuminata*	103975947	2,651	2	6	40.4
		Musa balbisiana		2,658		5	
		Musa acuminata	103989799	2,790	6	6	93.5
		Musa balbisiana		2,685		6	

Note. % Similarity value was obtained through global pairwise alignment with EMBOSS-Needle

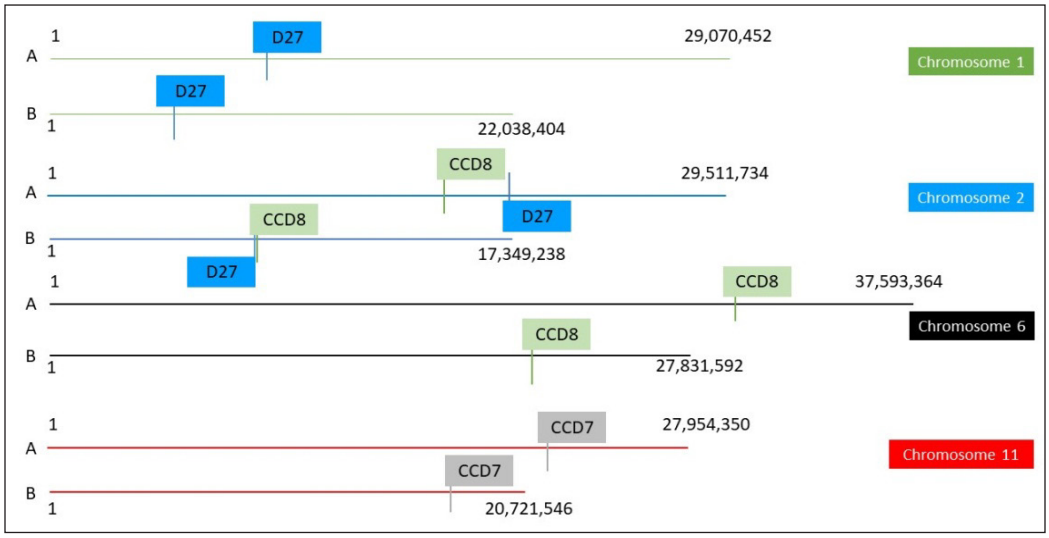

Figure 1. Positions of *D27*, *CCD7*, dan *CCD8* genes at chromosomes of DH-Pahang (labelled A) and PKW (labelled B)

Note. Each chromosome is given different colours, and its sequence length is labelled. Each gene is labelled with different colours: *D27* in blue, *CCD7* in grey, and *CCD8* in green

The search and prediction of genes based on nucleotide sequences in GenBank and genetic sequences of *A. thaliana* results in identical or similar nucleotide sequences and have similar positions in both DH-Pahang's genome and PKW's. However, there are differences such as the prediction results of the gene *D27* based on *A. thaliana* can only be found on chromosome 1; meanwhile, according to the prediction results based on the reference sequence from GenBank, the *D27* gene was found on chromosomes 1 and 2.

Genetic Structure Comparison of *D27* Gene in DH-Pahang and PKW

Figure 2 shows the exon-intron structure (part-a), the protein domain structure (part-b), and the putative protein motif structure (part-c) of *D27* in DH-Pahang and PKW (motif details available in Table S1).

D27 genes in DH-Pahang were retrieved from GenBank. *D27* was found in chromosomes 1 and 2, with GeneID 103986218 for *D27* at chromosome 1 and 103976367 for *D27* at chromosome 2. The nucleotide sequence length of the *D27* gene at chromosome 1 of DH-Pahang was 3,532 base pairs (bp) with seven exons, and at chromosome 2 was 2,891 bp with five exons. *D27* could also be found at chromosome 1 and 2 of PKW's whole genome sequence. The nucleotide sequence length of the *D27* gene at chromosome 1 was 2,891 bp and at chromosome 2 was 2,897 bp. In *A. thaliana*, the number of exons in the *D27*-like gene (GeneID: 838334) was seven (Waters et al., 2012). Sequence alignment between the *D27* gene in chromosome 1 of DH-Pahang and PKW resulted in a similarity percentage between the two sequences of 41%. *D27* gene in chromosome 2 of DH-Pahang and

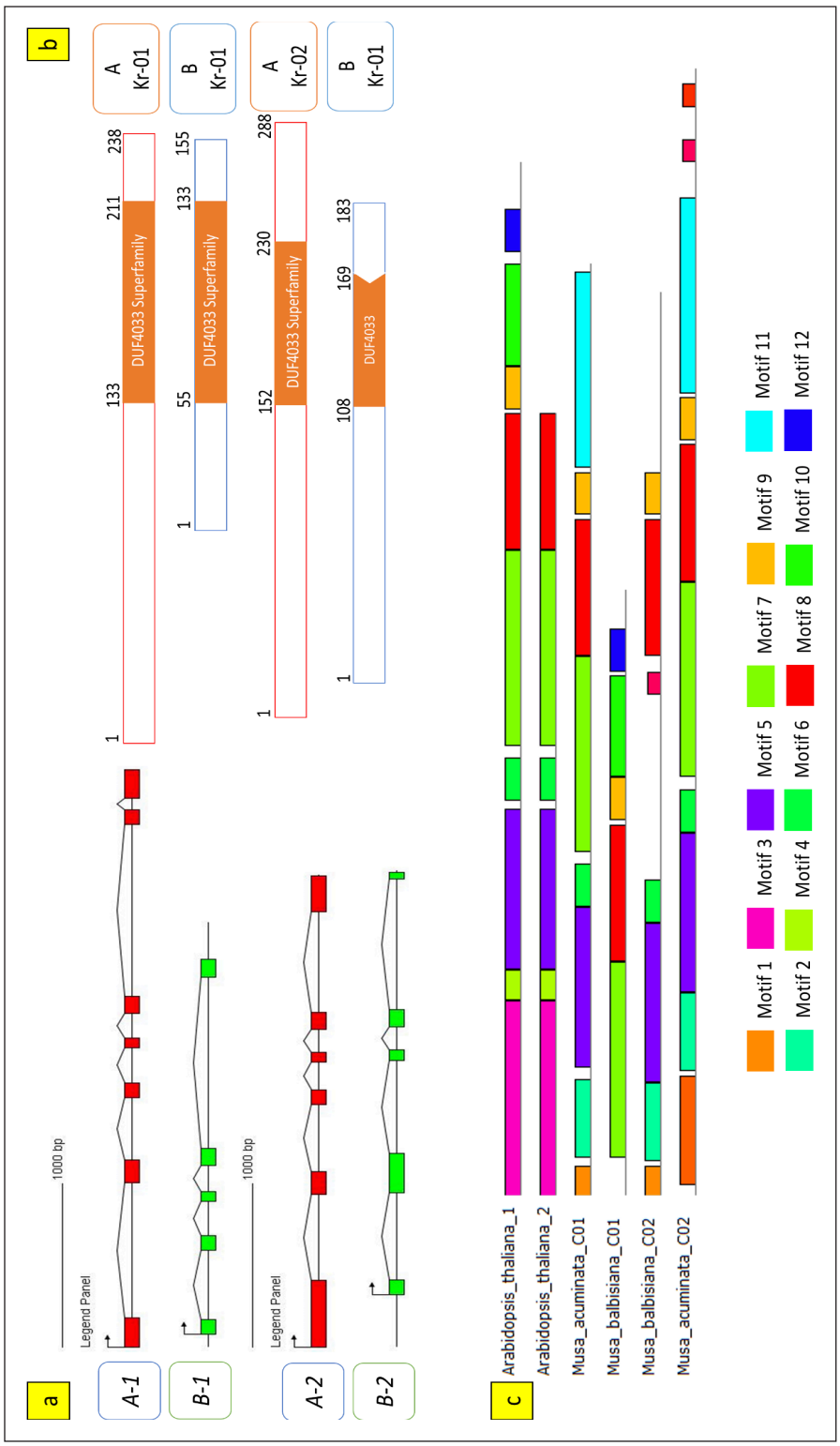

Figure 2. Comparison of exon-intron structure, protein domain and motif structure in *D27*
Note. a) Comparison of exon-intron structures of *D27* gene in chromosome 1 and 2 DH-Pahang (*Musa acuminata*) (A-1, A-2) and chromosome 2 PKW (*Musa balbisiana*) (B-1, B-2); b) Domain structure of D27 putative protein as the product of the gene in chromosome 1 and 2 in DH-Pahang (A) and PKW (B). Kr-01: chromosome 1 and Kr-02: chromosome 2. Numbers at the side indicate the number of amino acid residue in a sequence; **c)** Motifs of D27 putative protein sequences in DH-Pahang, PKW, and *Arabidopsis thaliana* predicted with MEME-Suite

Table S1
Motif consensus sequence of D27 putative protein and result of motif search with InterProScan

D27 Motif	E-value	Length (aa)	Available at the database?	Motif consensus sequence
1	2.4e+003	8	No	MELGQQRP
2	3.4e-004	20	No	RKLLSSVVEARVQTEEKMVA
3	1.7e-016	50	No	MNTKLSLSQTKIFTFTTWFNDTRSGLDRRSSISPTLCSK-PVYSGKLKAAK
4	3.7e+003	8	No	ETARIETS
5	2.7e-325	41	Yes**	ATEKTVYKDNWFDKLAIGYLSRNLQEASGMKNEKDGYESLI
6	5.7e-038	11	No	EAAJMISRLFD
7	2.0e-406	50	Yes**	QQELVIQALERAFPSYILTMIKVMLPPSKFSREYFAAFT-TIFFPWLVGPC
8	6.9e-395	35	Yes*	EVRESEVDGRKEKNVVYIPKCRFLESTNCVGMCTN
9	3.4e-104	11	No	CKIPSQKFIQD
10	6.8e-003	26	Yes***	SLGMPIYMEPDFEDLSCEMIFGRZPP
11	1.9e-424	50	Yes*	LGMPVYMSPNFEDMSCEMIFGQQPPEDDPALKQPCYRTK-CIAKQNHGVNC
12	4.0e+000	11	No	DDPALKQPCYH

Note. *) Motif: Beta-carotene isomerase D27-like (IPR038938); in domain of unknown function DUF4033 (IPR025114); **) Motif: Beta-carotene isomerase D27-like (IPR038938); ***) Motif in domain of unknown function DUF4033 (IPR025114)

PKW was 97.2% similar; hence it could be predicted that *D27* in chromosome 2 as more conserved between two different *Musa* species.

Domain and Motif of D27 Putative Protein and Comparison between DH-Pahang and PKW

D27 putative protein's amino acid sequences in DH-Pahang were retrieved from GenBank (accession number XP_009402431 for D27 in chromosome 1 and XP_018677614 for D27 in chromosome 2).

D27 putative protein's amino acid sequences in PKW were predicted through their respective nucleotide sequences using FGENESH. All D27 proteins have the domain DUF4033, and they are in the protein family DUF4033. DUF4033 (domain of unknown function 4033) is a domain with a function that has not been characterized. This domain can be found in bacteria and eukaryotes, 80 amino acids-long (Marchler-Bauer et al., 2017). Most DUFs are highly conserved, and this suggests their essential role in biological function. However, most DUFs are non-essential, so their biological role became difficult to be determined. DUFs are believed to be only needed under certain conditions (Häuser et al., 2012).

Based on motif prediction with MEME-Suite of 16 D27 protein sequences from 13 plant species, protein motifs found are shown in Figure 2c. The motif sequence consensus was then used as a query searching for the types of motifs and protein families

containing these motifs using InterProScan. Five motifs were found in the database. The existence of unidentified motifs could be estimated as novel motifs that need to be studied further regarding their role in this protein's function. Based on the results of the search for motifs in InterProScan, the five motifs found in the database were those found in the D27-like β-carotene isomerase protein family (IPR0389038) and were incorporated in the DUF4033 domain component.

The motif structure of each D27 protein sequence from DH-Pahang, PKW, and *A. thaliana* are as shown in Figure 2c. All D27 proteins in DH-Pahang, PKW, and *A. thaliana* had motifs that form the B-carotene isomerase domain (motifs 5, 7, 8, and 10). Motifs 1 and 2 were only found in DH-Pahang and PKW, while motifs 3 and 4 were only found in *A. thaliana*. Motif 11 appeared only on *M. acuminata*. In general, the genus *Musa* has motifs 1, 2, 5, 6, 7, 8, and 9. However, in the putative protein sequence D27 *M. balbisiana* from chromosome 1, the only motifs shared by other *Musa* species were motifs 7, 8, and 9. This could be because the putative protein sequences were cut off at the front and back because the protein sequence prediction using FGENESH was not perfect.

CCD7 Genetic Structure Comparison between DH-Pahang and PKW

Figure 3 shows the exon-intron structure (part a), the protein domain structure (part b), and the putative protein motif structure (part c) in *CCD7* gene in DH-Pahang and PKW. Detailed motif consensus sequence of CCD7 putative protein and result of motif search are available in Table S2.

The *CCD7* gene in DH-Pahang was obtained from GenBank. This gene was found on chromosome 11, with GeneID 103972006. The *CCD7* gene on chromosome 11 DH-Pahang had a sequence length of 3,768 bp. The *CCD7* gene BLAST results from the PKW whole genome sequence were found on chromosome 11, with a sequence length of 2,897 bp. The similarity of *CCD7* genes in *M. acuminata* and *M. balbisiana* was 95.4%. This indicates that this gene was sufficiently conserved in two plant species in the same genus. This was in accordance with the study by Ahrazem et al. (2016): that *CCD7* is a well-conserved gene between plant species; this gene has a function crucial to the organism's survival, hence its structure is maintained. The number of exons in *CCD7* DH-Pahang was 7, while in PKW, there were 6. This was in accordance with the results of research by Wang et al. (2017), the number of *CCD7* exons of various plants ranged from 5 to 7.

Domain and Motif of CCD7 Putative Protein and Comparison between DH-Pahang and PKW

DH-Pahang's CCD7 putative protein sequence was retrieved from GenBank (accession number XP_009384463). The putative protein sequence of the *CCD7* gene on PKW was obtained from gene prediction results in FGENESH. CCD7 proteins belong to the RPE65 domain, and all of them belong to the RPE65 protein superfamily. The RPE65 domain (retinal pigment epithelium

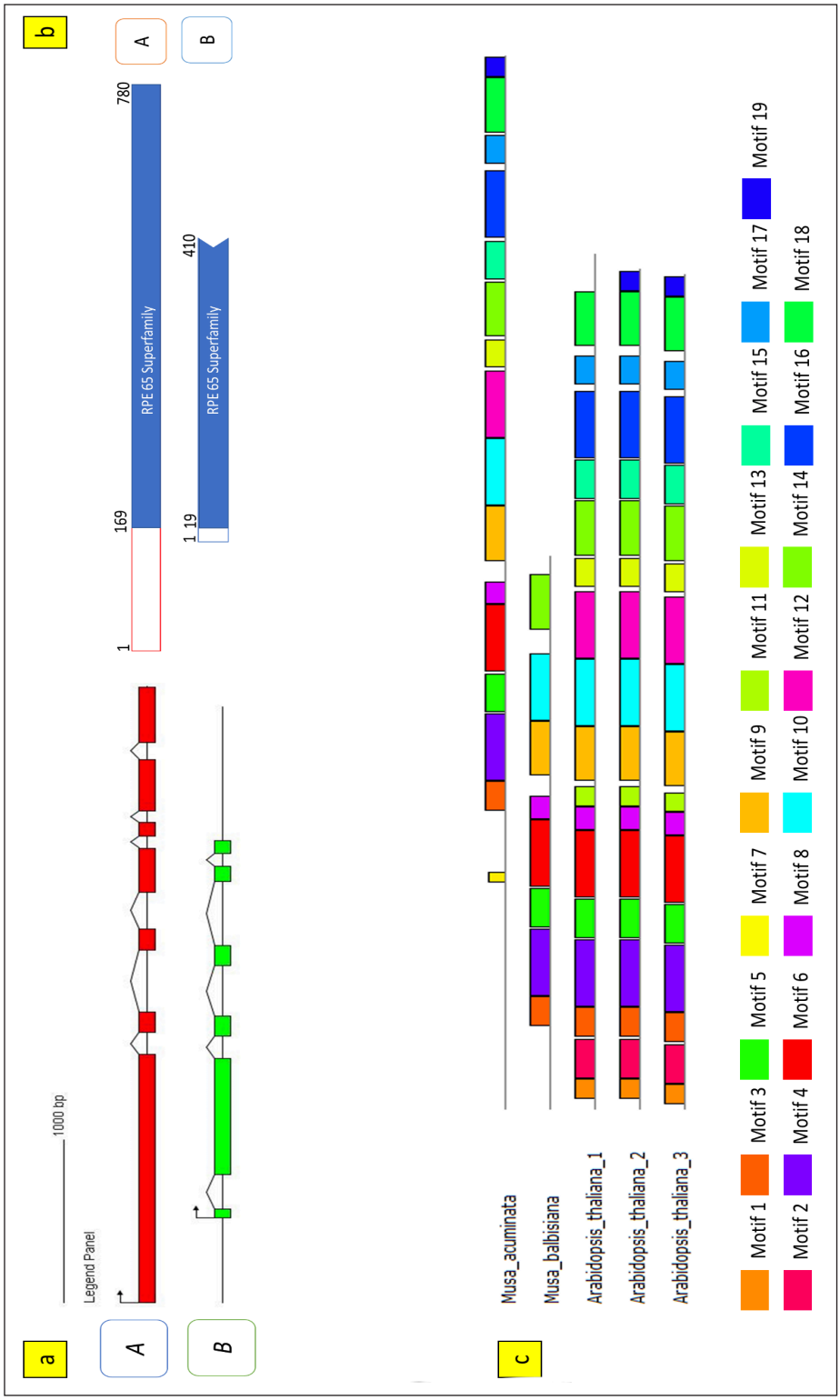

Figure 3. Comparison of exon-intron structure, protein domain, and motif structure in *CCD7*
Note. a) Comparison of exon-intron structures of *CCD7* gene in chromosome 11 DH-Pahang (*Musa acuminata*) (A) and PKW (*Musa balbisiana*) (B); **b)** Domain structure of CCD7 putative protein as the product of the gene in DH-Pahang (A) and PKW (B). Numbers at the side indicate the number of amino acid residue in a sequence; **c)** Motifs of CCD7 putative protein sequences in DH-Pahang, PKW, and *Arabidopsis thaliana* predicted with MEME-Suite

Table S2
Motif consensus at CCD7 putative protein and search result with InterProScan

CCD7 Motif	E-value	Length (aa)	Available at the database?	Motif consensus sequence
1	2.5e-034	15	No	IPPKLLPPAKLPPTH
2	3.9e-052	29	No	HGQTNLPLAESKCKDSWSMPDDNMVRLGT
3	3.7e-186	22	No	PDSTSAAFWDYQFLFVSQRSET
4	1.1e-515	50	Yes*	AEPVVLRVVEGSIPVDFPSGTYYLAGPGLFTDDHGSTVH-PLDGHGYLRAF
5	1.3e-176	29	No	IDGSSGQVKFSARYVETEAQREERDPVTG
6	5.2e-620	50	Yes*	WRFTHRGPFSVLKGGKRVGNTKVMKNVANTSVLRWGGRLL-CLWEGGDPY
7	2.7e-018	8	-	MQAKPCHN
8	1.5e-080	17	No	IDSRTLDTVGKFDLIGN
9	2.8e-375	41	No	FLDVAAHLLKPILYGVFKMPPKRLLSHYKIDARRNRLLMVS
10	1.3e-615	50	No	CNAEDMLLPRSNFTFYEFDSNFELLQKKEFVIPDHLMIH-DWAFTDTHYIL
11	4.1e-011	15	No	GCESCDDDDSSDRDL
12	6.4e-367	50	No	FGNRIKLDIPGSLLAVCGLSPMISALSVNPSKPSSPI-YLLPRFSDKEARG
13	5.1e-185	21	No	WRVPIEAPSQLWVLHVGNAFE
14	5.8e-466	41	No	DENGNLNIQJQASGCSYQWFNFQKMFGYBWQSGKLDPSFMN
15	5.0e-181	29	No	EEGEEKLLPHLVQVSINLDSTGNCTRCSV
16	3.2e-393	50	Yes*	LSNQWNKPADFPAINPDFSGRKNKYVYAATSSGSRR-FLPHFPFDSVVKLB
17	1.6e-145	21	No	VRTWSAGARRFIGEPVFVPRG
18	6.2e-369	41	Yes*	EDDGYILVVEYAVSTQRCYLVILDAKKIGEKBAVVARLEVP
19	2.1e-113	15	No	KHLTFPLGFHGFWAD

Note. *) Carotenoid oxygenase protein family (IPR004294); domain RPE65 (PF03055). Sequence written in red font was not checked at InterProScan because its length is less than ten amino acids

protein) is a domain belonging to a protein family widely expressed in the retinal pigment epithelium. This protein family also consists of enzymes that can cut neoxanthin in plants, and lignostilbene-alpha, β-dioxygenase enzymes in bacteria. The CCD protein family is characterized by the presence of the RPE65 domain (Marchler-Bauer et al., 2017). Neoxanthin is a form of a carotenoid compound.

Based on motif prediction of 15 CCD7 amino acid sequences from 13 plant species, protein motifs found are shown in Figure 3c. The motif sequence consensus is then used as a query searching for the types of motives and protein families containing these motifs using InterProScan. Four motifs were found in the database. The existence of motifs which functions have not been identified could be estimated as novel motifs

that need to be studied further regarding their role in this protein's function. Based on the results of the search for motives in InterProScan, the four motifs found in the database were those found in the carotenoid oxygenase protein family (IPR004294) and incorporated in the RPE65 domain component.

Meanwhile, protein prediction results concluded that proteins in the family carotenoid oxygenase are proteins involved in the reduction-oxidation process (GO: 0055114) and have a specific molecular function: oxidoreductase activity (GO: 0016702). The motif for each CCD7 amino acid sequence in DH-Pahang, PKW, and *A. thaliana* is shown in Figure 3c. All CCD7 putative proteins have the carotenoid dioxygenase motif (motifs 4, 6, 16, and 17). Motifs 1 and 2 were only found at *A. thaliana*, and motif 7 was only found at *M. acuminata*. Both *M. acuminata* and *M. balbisiana* have the motifs 3, 4, 5, 6, 8, 9, 10, and 11. However, in *M. acuminata,* some motifs were not found at the CCD7 amino acid sequence of *M. balbisiana*. This could be because the putative protein sequences were cut off at the front and back because the protein sequence prediction using FGENESH was not perfect.

CCD8 Genetic Structure Comparison between DH-Pahang and PKW

Figure 4 shows the exon-intron structure (part a), the protein domain structure (part b), and the putative protein motif structure (part c) of *CCD8* in DH-Pahang and PKW. Detailed motif consensus sequence of CCD8 putative protein and result of motif search are available in Table S3.

The *CCD8* gene in DH-Pahang was retrieved from GenBank. This gene was found on chromosomes 2 and 6, with GeneID 103975947 for *CCD8* on chromosome 2 and 103989799 for *CCD8* on chromosome 6. The *CCD8* gene on chromosome 2 DH-Pahang had the length of 2,651 base pairs, and on PKW 2,123 base pairs. The *CCD8* gene from BLAST resulted from the PKW whole genome sequence was also found on two chromosomes: chromosome 2 and chromosome 6 from PKW. The number of exons in the *CCD8* gene on chromosome 2 DH-Pahang was 6, while in PKW, there were 5. Sequence alignment analysis showed the similarity between the *CCD8* gene on chromosome 2 DH-Pahang and PKW was 40.5%. The *CCD8* gene on chromosome 6 DH-Pahang had 2,790 base pairs, while the *CCD8* gene from chromosome 6 had 2,685 base pairs. The number of exons in the *CCD8* gene from chromosome 6 DH-Pahang and PKW was five. After sequence alignment between the *CCD8* gene on chromosome 6 DH-Pahang and PKW, the percentage values of identity and similarity between these two sequences were 93.5%. The number of exons in the *CCD8* genes in DH-Pahang and PKW was five to six; consistent with the results of a study by Batra et al. (2019) regarding *CCD8* genes and proteins in seven monocot and dicot species: the number of exons in the *CCD8* gene generally ranges from four to seven.

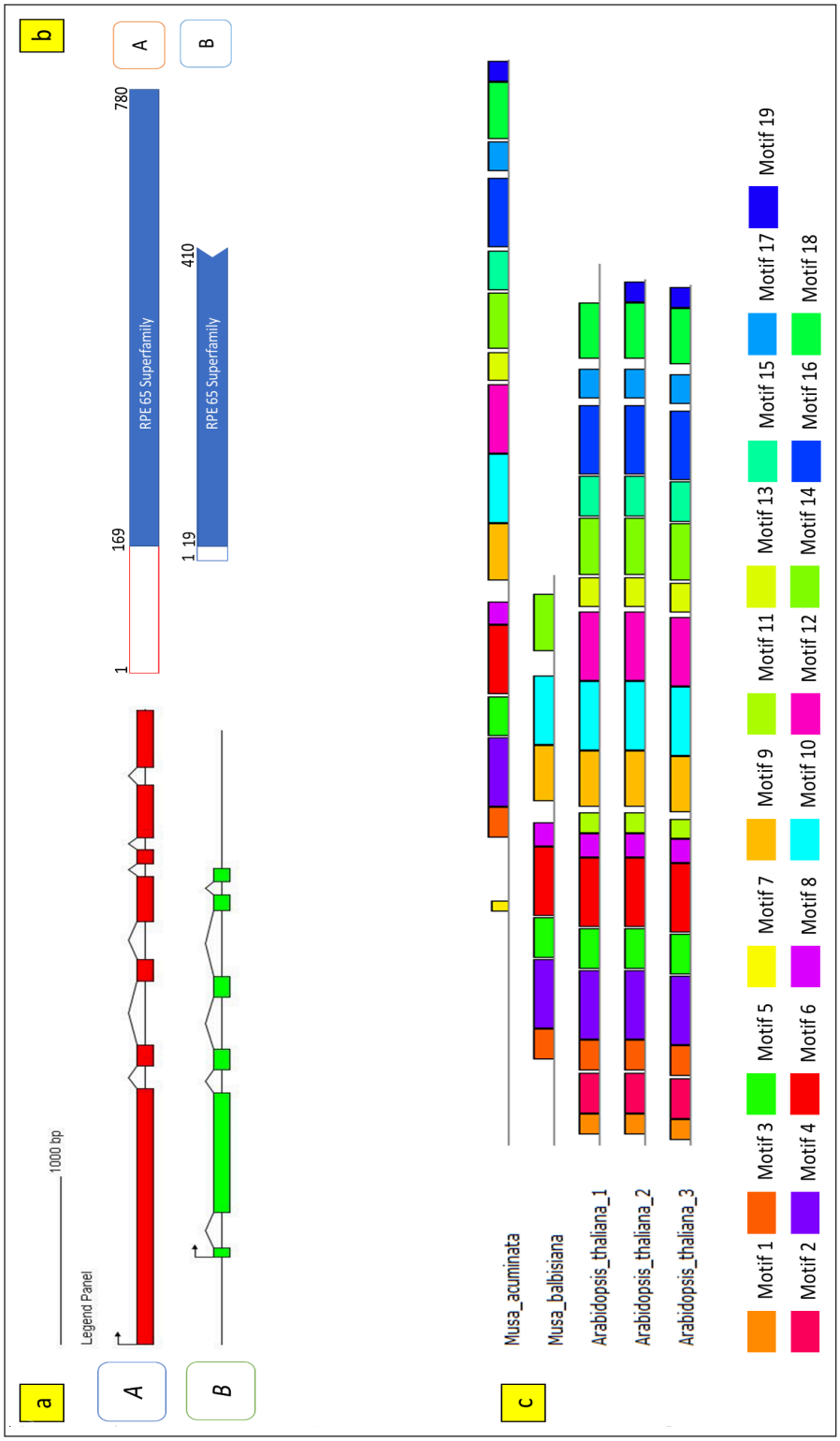

Figure 4. Comparison of exon-intron structure, protein domain, and motif structure in *CCD8*

Note. a) Comparison of exon-intron structures of *CCD8* gene in chromosome 2 and 6 in DH-Pahang (*Musa acuminata*) (A-2, A-6) and chromosome 2 and 6 in PKW (*Musa balbisiana*) (B-2, B-6); b) Domain structure of CCD8 putative protein as the product of the gene in DH-Pahang (A) and PKW (B). Kr-02: chromosome 2 and Kr-06: chromosome 6. Numbers at the side indicate the number of amino acid residue in a sequence; **c)** Motifs of CCD8 putative protein sequences in DH-Pahang, PKW, and *Arabidopsis thaliana* predicted with MEME-Suite

Table 3
Motif consensus sequence of CCD8 putative protein and result of motif search with InterProScan

CCD8 Motif	E-value	Length (aa)	Available at the database?	Motif consensus sequence
1	1.9e-028	50	No	TQFSSPKAHASHAHVAVSTRPGSVYSGNSIGDAVNK-SKPHVPGGLRARRV
2	5.1e-005	21	No	ETQVAPEPQPEPEKGGGEERK
3	6.1e-020	21	No	RRPAESVRASVATEPRPTVPS
4	2.6e+000	15	No	FDPAVETKQDVGSGR
5	1.9e-002	20	No	MASTLFSPPLQPTAIFPSTR
6	2.1e+003	7	-	FDTICRR
7	1.1e-588	50	Yes*	AWTSIRQERWEGELVVZGEIPLWLNGTYLRNGPGL-WNIGDYNFRHLFDGY
8	3.0e-523	50	Yes**	TLVRLHFENGRLIAGHRQIESEAYKAAKKNKRL-CYREFSEVPKPDNFLAY
9	1.3e-012	6	-	GELASL
10	7.4e-588	50	Yes*	FSGASLTDNANTGVVKLGDGRVVCLTETIKGSIQID-PDTLETIGKFEYSD
11	1.9e-569	50	Yes**	GLIHSAHPIVTESEFLTLLPDLVRPGYLVVRMEPGS-NERKVIGRVNCRG
12	3.4e-666	50	Yes*	WVHSFPVTEHYVVVPEMPLRYCAQNLLRAEPTPLYK-FEWHPESGSFMHVM
13	1.5e-331	29	No	CKASGKIVASVEVPPYVTFHFINAYEEKD
14	1.2e-580	50	Yes**	TAIIADCCEHNADTTILDKLRLQNLRSFSGEDVLP-DARVGRFRIPLDGSP
15	1.3e-060	11	No	GELEAALDPEE
16	1.8e-677	50	Yes*	HGRGMDMCSINPAYLGKKYRYAYACGAQRPCNF-PNTLTKIDLVEKKAKNW
17	5.9e-094	11	No	GAVPSEPFFVA
18	1.9e-589	50	Yes*	RPGATEEDDGVVISMVSDKNGEGYALLLDGSTFEEI-ARAKFPYGLPYGLH
19	8.1e-050	6	-	GCWVPK

Note. *) Carotenoid oxygenase protein family (IPR004294) and domain RPE65 (PF03055); **) Carotenoid oxygenase protein family (IPR004294). Sequences written in red font were not checked at InterProScan because their lengths are less than ten amino acids

Domain and Motif of CCD8 Putative Protein and Comparison between DH-Pahang and PKW

The amino acid sequences for the putative protein CCD8 from DH-Pahang were obtained from GenBank (accession number XP_009389368 for chromosome 2 and XP_009407022 for chromosome 6). The amino acid sequences for the putative protein of the CCD8 gene on chromosomes 2 and 6 PKW were obtained from gene prediction results in FGENESH. All CCD8 proteins belong to the RPE65 domain, and like other CCD proteins, they all belong to the RPE65 protein superfamily (Marchler-Bauer et al., 2017). The RPE65 domain

(retinal pigment epithelium protein) is the domain that belongs to the entire CCD protein family. This domain is characteristic of enzymes involved in apocarotenoid biosynthesis, the intermediate product of the synthesis of strigolactone from β-carotene (Batra et al., 2019).

Based on the prediction of protein motifs of 16 CCD8 protein sequences from 13 plant species, the protein motifs found are shown in Figure 4c. The motif sequence consensus was then used as a query in searching for the types of motifs and protein families containing these motifs using InterProScan. Four motifs were found in the database. The existence of motifs which functions had not been identified could be predicted as novel motifs that needed to be studied further regarding their role in this protein's function. Based on the motif search results in InterProScan, the eight motifs found in the database were those found in the carotenoid oxygenase protein family (IPR004294) and incorporated in the RPE65 domain component.

Meanwhile, protein prediction results concluded that proteins in the carotenoid oxygenase family were proteins involved in the reduction-oxidation process (GO: 0055114). They have a particular molecular function of oxidoreductase activity (GO: 0016702). This corresponds to the putative protein sequence CCD7, a protein in the same family as the CCD8 protein (Wang et al., 2017). The motif structures of each CCD8 protein sequence from DH-Pahang, PKW, and *Arabidopsis thaliana* are shown in Figure 4c. All CCD8 putative proteins had a carotenoid oxygenase motif (motifs 7, 8, 10, 11, 12, 14, and 16). There were specific motifs only in monocot or dicot plant species: motifs 1 and 2 were only found in *A. thaliana*. In contrast, motifs 3 and 4 were only found in putative protein products CCD8 from chromosome 6 DH-Pahang and PKW. Motif 5 was found only in *M. acuminata*. In general, CCD8 putative protein sequences in the genus *Musa* had motifs 3, 5, 7, 8, 9 to 19. However, in the putative protein sequences of CCD8 chromosome 2 *M. balbisiana*, there were no motifs before motif 7, and this could be because the putative protein sequences were cut off at the front and back because the protein sequence prediction using FGENESH was not yet perfect. The difference between the motifs in *M. acuminata* and *M. balbisiana* could also be caused by the mutation process of insertion and deletion, along with the evolutionary process of the two species.

Phylogenetic Relations of Amino Acid Sequences of D27, CCD7, and CCD8 Putative Proteins in DH-Pahang, PKW, and Other Plant Species

A phylogenetic tree was built from putative protein D27 on chromosome 1 and 2 DH-Pahang and putative protein D27 from PKW and other plants; as shown in Figure 5.

Figure 5 shows that the putative protein D27 from chromosome 1 DH-Pahang (*M. acuminata*) was present in the same clade as putative protein D27 from chromosome 1 PKW (*M. balbisiana*); the same goes for putative protein D27 from chromosome 2. Putative protein D27 from the genus *Musa*

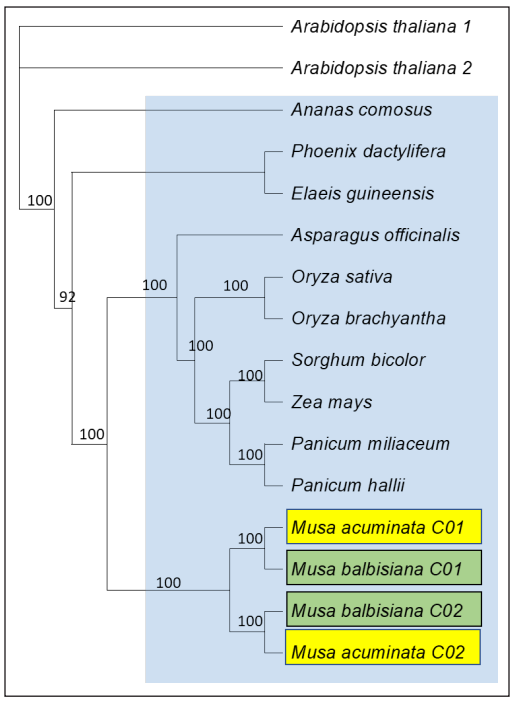

Figure 5. Phylogenetic tree based on putative protein sequences of D27 DH-Pahang (*Musa acuminata*), PKW (*Musa balbisiana*), and D27 protein from other plants

Note. C01 = chromosome 1, C02 = chromosome 2. The blue square indicates monocot plants. The tree was constructed using the Bayesian algorithm with MrBayes software, nGen = 20,000, and mutation rate = invgamma

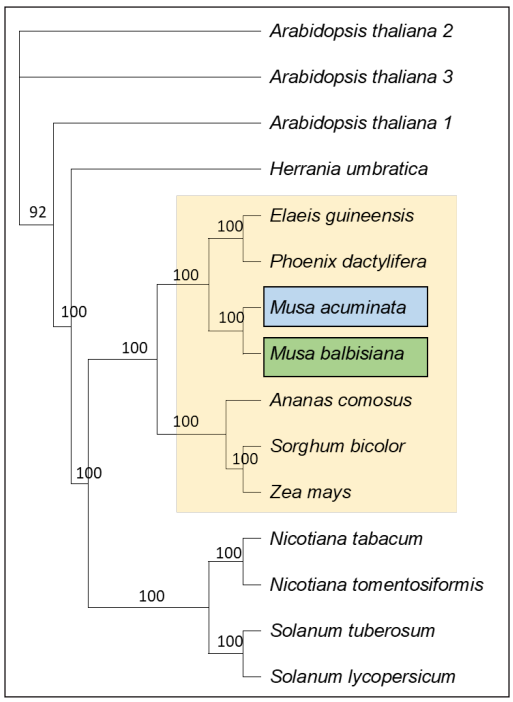

Figure 6. Phylogenetic tree based on putative protein sequences CCD7 DH-Pahang (*Musa acuminata*), PKW (*Musa balbisiana*), and CCD7 protein from other plants

Note. The yellow square indicates monocot plants. The tree was constructed using the Bayesian algorithm with MrBayes software, nGen = 20,000, and mutation rate = invgamma

was found in a separate clade from other species, but still had a common ancestor with monocot species such as species in the genus *Panicum* and *Oryza*, as well as species such as *Sorghum bicolor* and *Asparagus officinalis*.

The phylogenetic tree based on the putative protein sequence CCD7 in DH-Pahang, PKW, and other plants is shown in Figure 6. The CCD7 putative protein in DH-Pahang (*M. acuminata*) was present in the same clade as the putative protein CCD7 PKW (*M. balbisiana*). The *Musa* clade has a common ancestor with the clade *Elaeis guineensis* - *Phoenix dactylifera*. This clade was a sister group of clades of monocot plants such as *Ananas comosus*, *Sorghum bicolor*, and *Zea mays*. Meanwhile, other species were dicot plants; for example, the genera *Nicotiana* and *Solanum* belonging to the Solanaceae family and were separated from the clades of monocot plants.

The phylogenetic tree based on the putative protein sequence CCD8 from chromosomes 2 and 6 in DH-Pahang, PKW, and other plants is shown in Figure 7.

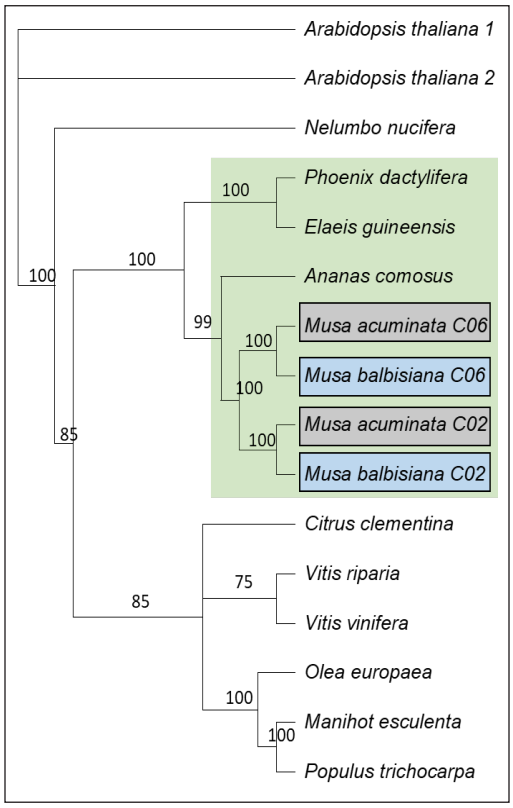

Figure 7. Phylogenetic tree based on putative protein sequences CCD8 DH-Pahang (*Musa acuminata*), PKW (*Musa balbisiana*), as well as CCD8 protein from other plants

Note. C02 = chromosome 2, C06 = chromosome 6. The green square indicates monocot plants. The tree was constructed using the Bayesian algorithm with MrBayes software, nGen = 20,000, and mutation rate = invgamma

The putative protein CCD8 from chromosome 2 DH-Pahang (*M. acuminata*) was in the same clade as the respective protein in PKW (*M. balbisiana*); likewise, putative protein CCD8 from chromosome 6. The putative protein CCD8 from the genus *Musa* was in the same clade as *Ananas comosus*, a monocot plant. These three species also belonged to the same clade as *Elaeis guineensis* and *Phoenix dactylifera*, both monocot plants. This monocot plant group was separate from other plants, which included the dicot group.

Based on this phylogenetic analysis results, it can be concluded that all putative protein sequences of DH-Pahang and PKW were the most similar to monocot plant species.

The putative protein sequences D27, CCD7, and CCD8 of the monocot species were in separate clades with dicot species. This suggested that the *D27*, *CCD7*, and *CCD8* genes diverged at an early stage in monocots and dicots' evolutionary history. This was consistent with research conducted by Batra et al. (2019), in which they also found that amino acid sequences of the *CCD8* gene of seven monocot species and eight dicot species showed a divergence between monocot-dicot.

CONCLUSION

This study concluded that the *D27*, *CCD7*, and *CCD8* genes in genomes A and B have different nucleotide sequence lengths and putative protein and different numbers and positions of exon-introns. Then, putative protein D27 has the DUF403 domain, whereas putative protein CCD7 and CCD8 have the RPE65 domain as the marker domain of each family.

The next steps are to isolate and sequence the *D27*, *CCD7*, and *CCD8* genes in DH-Pahang and PKW; and characterize their protein products in further research. In addition, there were still unidentified putative

protein motifs, and they may be essential motifs as markers and differentiators of the D27, CCD7, and CCD8 proteins in *Musa* compared to other plant species.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Effect of Screen House on Disease Severity and Coat Protein Diversity of *Begomovirus*-infected *Capsicum frutescens* L. 'Cempluk' from Indonesia

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ABSTRACT

Chili can be infected by *Begomovirus* through whiteflies (*Bemisia tabaci*) serving as a vector insect. *Begomovirus* infection causes dwarf plants and yellow curly leaves. The molecular detection of *Begomovirus* coat protein gene may serve as a preliminary identification of *Begomovirus*. This study was conducted to observe the differences in the symptom severity of *Begomovirus* infection in chilies (*Capsicum frutescens* L. 'Cempluk') planted inside and outside a screen house. This study also observed whether or not using a screen house in chili farming affects the diversity of the coat protein of *Begomovirus*. Symptom observation and sampling were conducted in Madurejo, Prambanan, Sleman. Molecular detection was performed by amplifying the coat protein (CP) gene using the universal primer Krusty and Homer. Results showed 7 plant samples with DNA bands ± 550 bp and confirmed that the plants were positively infected with *Begomovirus*. The amplified bands were purified and sequenced. The nucleotide sequences were analyzed using BLASTn, followed by phylogenetic analysis using MEGA. Planting chili in the screen house resulted in low disease severity and good crop conditions. The coat protein sequence showed different strains of *Begomovirus* infected the chili plants inside and outside the screen house. *Pepper yellow leaf curl Indonesia virus* (PepYLCIV) was found inside the screen house while PepYLCIV [Ageratum] was dominant outside the screen house. Both strains are closely related to other *Pepper yellow leaf curl virus* (PepYLCV) from various regions in Indonesia. Optical

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manipulation using an ultraviolet screen or screen house was effective in reducing *Begomovirus* infection and improving plant performance.

Keywords: *Begomovirus*, coat protein, disease severity, *Pepper yellow leaf curl Indonesia virus*, plant defense mechanism, plant hormones

INTRODUCTION

Yellow leaf curling caused by *Begomovirus* in chili has long been a problem in Indonesia and has caused crop failure in various regions. Yellow leaf curling in chili was first reported in 1999 in West Java as being caused by geminiviruses, mainly from the genus *Begomovirus* (Rusli et al., 1999). Since 2000, this disease has spread in Yogyakarta and Central Java and has reduced chili production (Sulandari et al., 2006).

Begomovirus-infected chilies are characterized by the presence of specific yellow mosaics on the leaves, curling of leaves from the edges, and stunted plant growth (Sulandari et al., 2006). *Begomovirus* infections are usually transmitted by whitefly (*Bemisia tabaci*), which is a viruliferous insect that feeds on plant phloem by injecting enzymes and secreting sap. It reduces plant strength or, in the case of severe attacks, kills the host (Horowitz et al., 2011; Kumar et al., 2017). The whitefly population increases in high humidity and causes crop failure. *Begomovirus* infection in chili plants may reduce harvest by 20%–100% (Setiawati et al., 2005).

Begomovirus is the largest genus in the *Geminiviridae* family. It infects a wide range of hosts, such as cultivated plants, weeds around plants, monocotyledonous plants, and dicotyledonous plants in tropical or temperate climates. Some of its strains that were reported to have infected chili and tomato plants were the *Tomato leaf curl Java virus* (Kon et al., 2006), *Pepper yellow leaf curl Indonesia virus*, *Tomato leaf curl Philippine virus* (Sakamto et al., 2005), *Ageratum yellow vein virus* (Tsai et al., 2009), and *Tomato yellow leaf curl Kanchaburi virus* (Kenyon et al., 2014). Its genome consists of ssDNA components DNA-A and DNA-B, and each genome size measures 2.5–2.8 kb (Snehi et al., 2011). *Begomovirus* is divided into two groups according to complete nucleotide sequences: bipartite and monopartite. The bipartite group has two types of ssDNA components, namely, DNA-A and DNA-B; the monopartite group has one circular homologous genomic DNA with DNA-A. Open reading frames (ORFs) in DNA-A and DNA-B in *Begomovirus* genomes produce several types of proteins with different functions. One of the most important ORFs is AV1/CP. This gene is responsible for expressing the protein coat that forms viral capsids. Furthermore, the CP gene regulates the transmission of the virus to whiteflies and host plants. The CP gene is the most conserved genome region among all genes/ORFs (Snehi et al., 2011; Wartig et al., 1997).

Begomoviruses tend to rejoin, thus causing new *Begomovirus* strains and new plant diseases in various host plants (Chakraborty et al., 2003; Varma & Malathi, 2003). Therefore, the detection of *Begomovirus* particles is needed to determine infected plants and find the source of infection. The assessment of viral diseases can be conducted by morphological observation and molecular detection. Symptom monitoring is a simple, fast, and inexpensive method for assessing the presence of *Begomovirus* infection. However, the results often vary due to subjective interpretations and effects of environmental conditions, hence the need to conduct reliable assessments, such as molecular detection (González-Pérez et al., 2011). The application of polymerase chain reaction (PCR) techniques to detect viral infections in plants is a sensitive, reliable, reproducible, and effective method, especially for large samples (Lopez et al., 2008). Previous studies reported the use of molecular methods to detect *Begomovirus* infections in chilies and eggplants (Maruthi et al., 2007), okra (Venkataravanappa et al., 2018), and tomatoes (Kusumaningrum et al., 2015). The present study was conducted to determine the effect of using screen houses in chili (*Capsicum frutescens* L. 'Cempluk') cultivation on the severity of *Begomovirus* infection and the diversity of *Begomovirus* coat proteins that cause yellow leaf curling in chilies using molecular methods.

METHODS

Morphological Observation and Sample Collection

Observation and sampling were performed in Madurejo, Sleman, Yogyakarta. Symptom observations were carried out in two study areas, namely, outside and inside a screen house in February–October 2018. A total of 469 and 41 chili plants (*Capsicum frutescens* L. 'Cempluk') were planted outside and inside the screen house. Virus infection in chili was classified into six scale groups on the basis of the plant height and scale of virus infection established by Srivastava et al. (2017) with a few modifications. The scale was as follows: 0: healthy plants, scale 1: yellow spot leaves, scale 2: yellow spots and moderately curved leaves, scale 3: yellow spots and curved leaves, scale 4: yellow and curly leaves, scale 5: fully yellow and curly leaves, stunted plants. Disease incidence (DI) was calculated using the following formula (Srivastava et al., 2017):

$$DI = \frac{Number\ of\ infected\ plants}{Total\ plants} \times 100\%$$

The scoring results were then converted into the disease severity (DS) index using the following formula to reveal DS (S. Islam et al.., 2010):

$$DS = \frac{\sum(ni \times Vi)}{Z \times N} \times 100\%$$

where, n_i = sum of plant in each score, V_i = score of symptoms, Z = value of highest

symptom, and N = total number of observed plants.

DNA Isolation and Amplification of Coat Protein Gene

DNA was extracted from chili leaf samples by using the commercial plant DNA extraction kit Illustra Phytopure™ and by following the processes described by Daryono and Natsuaki (2002) with slight modification. Furthermore, DNA was checked quantitatively using NanoDrop™ spectrophotometry with λ 260/280 nm. Universal primers for *Begomovirus* coat protein, namely, Krusty and Homer (Krusty [Forward]: 5′CCNMRDGGHTGTGARGGNCC′3; Homer [Reverse]: 5′SVDGCRTGVGTRCANGCCAT′3), were utilized to amplify the partial coat protein of *Begomovirus* by using the PCR thermal cycler (Revill et al., 2003). Infected plants show a DNA band at about ~550 bp in electrophoresis visualization. A PCR mix (25 µL) containing 12.5 µL PCR kit Bioline 2x MyTaq™ HS RedMix (Bioline, United Kingdom), 1.5 µL for each primer (10 pmol), 1.5 µL DNA samples (100 pmol), 0.5 µL $MgCl_2$, and 7.5 µL distilled water was used. The PCR reaction was started with initial denaturation at 95°C for 5 min; continued with 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and then followed by a final extension at 72°C for 5 min. The PCR results were analyzed using 2% of agarose gel, which was stained with FloroSafe DNA stain (First BASE, Singapore).

DNA Sequence Analysis

Positive PCR samples were sent to First BASE, Singapore for sequencing. Then, nucleotide sequences were assembled using GeneStudio™ software, followed by BLASTn analysis for comparing sequences with other coat protein gene sequences of *Begomovirus* in GenBank. A phylogenetic tree was constructed using MEGA 7.0 with 2,000 bootstraps and then edited manually.

RESULTS

Observed Symptoms on Leaves

The symptom observation of *Begomovirus* infection was focused on leaf morphology. However, some of the plants observed were found to be dead due to wilting rather than the *Begomovirus* infection. Seven and nine dead plants were found inside and outside the screen house, respectively. The differences in the responses of the plants inside and outside the screen house to the *Begomovirus* infection are detailed in Table 1. The results of the study showed that the incidence and severity of the disease was

Table 1
Response of chili to natural Begomovirus *infection*

	Symptomatic plants/ total plants	Disease incidence (%)	Disease severity (DS)
Chili inside screen house	25/41	60.9	42
Chili at field	455/464	97	53.8

greater in the chili plants outside the screen house than in those planted inside the screen house. The use of a screen house thereby reduced the *Begomovirus* infection because it protected the plants from whiteflies while minimizing the effects of environmental stress on plant development.

The severity of the morphological symptoms on the leaves of the chili plants was grouped into six categories according to the observed symptoms (Figure 1). This scale determination was based on the study of Srivastava et al. (2017). The most common morphological symptoms in the chilies planted outside the screen house were yellow and curved leaves (scale 3). Those observed in the chilies inside the screen house indicated relatively health plants despite the presence of yellow spots on the leaves (scale 1). The real difference was that no plant inside the screen house showed yellow and curly leaves (scale 4). The most severe symptoms observed in both locations were completely yellow and curly leaves and stunted growth.

Effect of *Begomovirus* Infection on Plant Height

One symptom of *Begomovirus* infection is stunted plant growth. Hence, plant height measurements were carried out on the plants in both study areas. The chilies planted outside the screen house were measured to be about 126–135 cm tall, whereas those planted in the screen house were taller with a height of 142–149 cm. Rank analysis in SPSS® software also showed a significant difference in the average heights of the chili plants outside and inside the screen house (Table 2). This condition supports the previous explanation that planting chilies in a screen house provides good plant conditions.

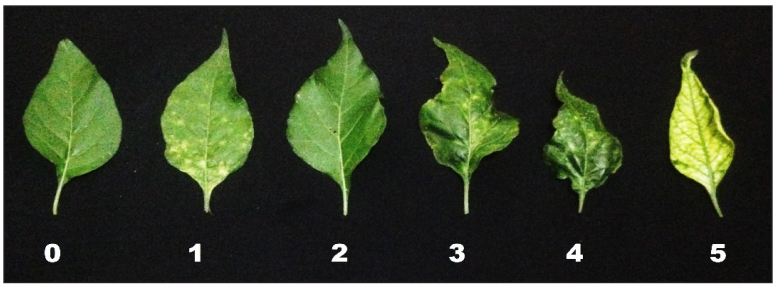

Figure 1. Morphological comparison of leaves based on the scale of virus infection: 0: healthy plants, 1: yellow spot leaves, 2: yellow spots and moderately curved leaves, 3: yellow spots and curved leaves, 4: yellow and curly leaves, 5: yellow and curly leaves, stunted plants, respectively

Table 2
Differences in average plant heights of chilies planted inside and outside the screen house

	Location	N	Mean rank	Sum of rank
Plant height	Screen house	34	303	10302
	Field	469	248.3	116454
	Total	503		

Molecular Diagnosis of *Begomovirus* Infection

Total DNA was extracted from 16 plant samples in the six infection scale groups; that is, eight samples were obtained from each of the two locations. A set of primers (Krusty and Homer) was used to amplify the CP gene region using the PCR technique. However, only 7 samples from the 16 samples were amplified; these samples showed a target DNA band at ±550 bp and were positively infected by *Begomovirus* (Figure 2). A total of five samples from outside the screen house produced the targeted bands, whereas only two samples from inside the screen house did. *Begomovirus* DNA was only found in the samples in the 3–5 scale; none was found in those in the 0–2 scale. This result indicated that the severity of the symptoms was correlated with the concentration of virus particles. The low severity of symptoms indicated low viral DNA concentrations and vice versa.

Diversity of Coat Protein Sequences

Each of the two samples from each location was sequenced to analyze the CP gene sequence. Then, the obtained sequences were analyzed using BLASTn to compare them with other nucleotide sequences in GenBank as the initial identification of *Begomovirus* strains. Table 3 shows that the two locations have the potential to be infected by the *Pepper yellow leaf curl Indonesia virus* with a CP percentage of similarity of >97%. According to the CP sequences, the samples from the screen house showed a close genetic relationship

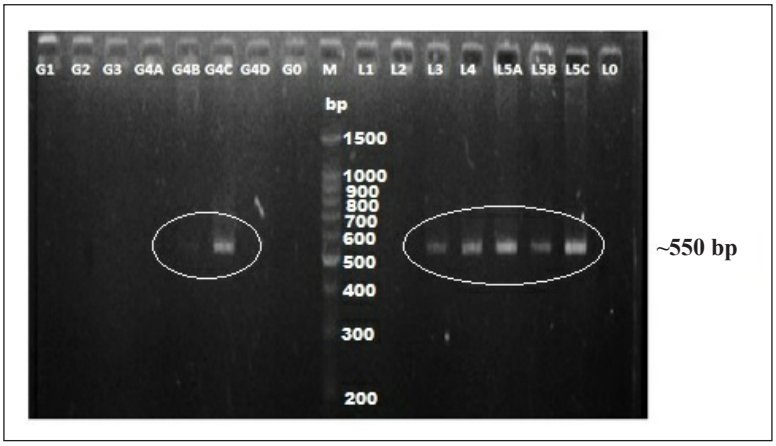

Figure 2. Electrophoresis visualization of CP gene amplification
Note. G: inside screen house samples; L: outside screen house samples; 0–6: infection scale

Table 3
Sequence analysis of Begomovirus *CP gene*

Sample	% Identity	Potential *Begomovirus* species	GenBank accession
Inside screen house	99.52	PepYLCIV	AB267834
Outside screen house	97.12	PepYLCIV [Ageratum]	AB267838

with the *Pepper yellow leaf curl Indonesia virus*, whereas the samples from outside the screen house showed CP sequences with 97.12% similarity to the *Pepper yellow leaf curl Indonesia virus* [Ageratum]. However, this analysis cannot be used for final identification as it may only serve as an indication of the prevalence of *Begomovirus* strains that cause curly leaves in chili 'Cempluk' planted inside and outside a screen house.

The nucleotide sequence of the CP gene from each location was compared with 22 nucleotide sequences from different virus strains to evaluate their genetic relationship. The genome samples used in the comparison came from several plants, such as *Capsicum frutescens*/chili, *Capsicum annum*, *Solanum lycopersicum*/tomato, and *Ageratum* (weeds). The analysis was performed by constructing a phylogenetic tree in MEGA 7.0. The phylogenetic tree showed that the two samples from the two locations are closely related to PepYLCIV isolated from various regions in Indonesia, such as West Sumatra, Banda Aceh, and Bogor (Figure 3). Begomoviruses detected in the plants from the inside and outside of the screen house were grouped into different clades. The samples isolated from inside the screen

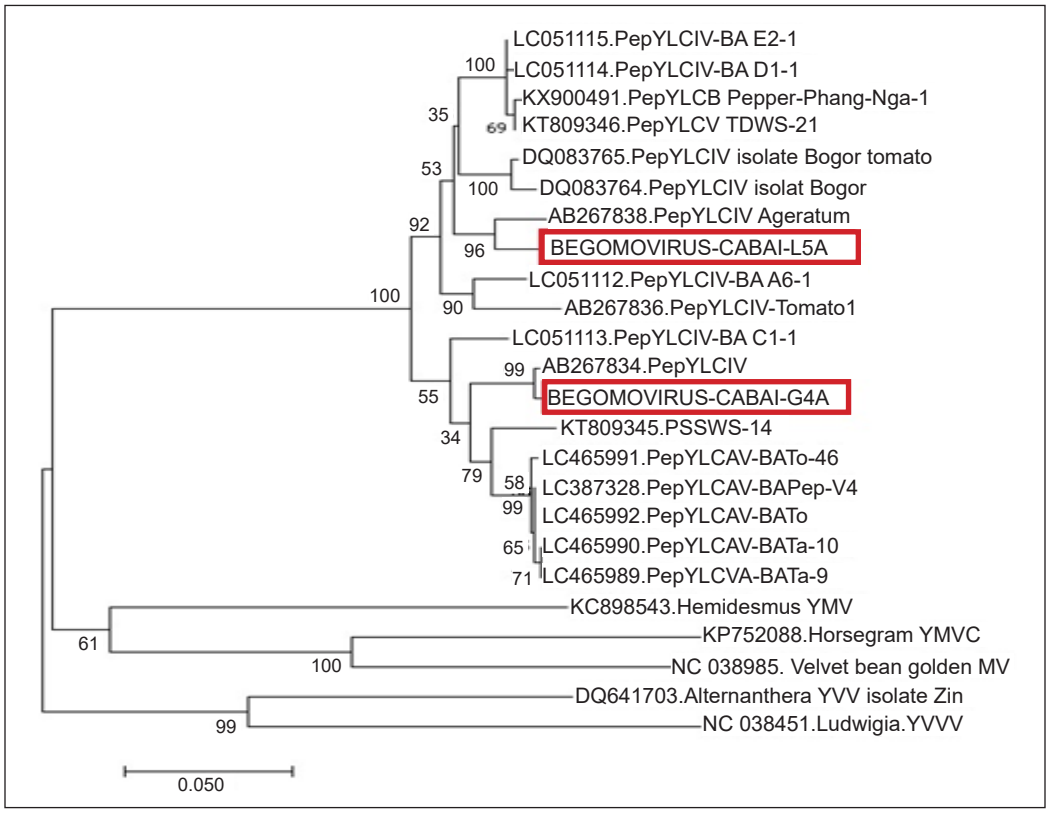

Figure 3. Phylogenetic tree of the partial CP gene of *Begomovirus* constructed using the neighbor joining algorithm with 2,000 bootstraps

house showed a close genetic relationship with PepYLCIV isolated from Bogor, West Java. The samples from outside the screen house showed a close genetic relationship with PepYLCIV [Ageratum] (Figure 3). These results indirectly indicated that the source of the *Begomovirus* infection outside the screen house came from nearby plantation crops or infected weeds.

DISCUSSION

Begomovirus infection in chili is a serious problem in Indonesian agriculture. Chili is one of the horticultural commodities with high economic value. Chili plants show several obvious symptoms when infected with *Begomovirus*; these symptoms include yellow spots on the leaf surface, vein clearing, thickening at the leaf bones, and cupping (Sulandari et al., 2006). Morphological observation is a simple way to identify the early symptoms of *Begomovirus* infection (González-Pérez et al., 2011). The results of symptom observations are usually converted into DI and DS to obtain a reliable estimation for plant response determination (Bock et al., 2015). The present study showed that DS inside the screen house was lower than that outside the screen house (Table 1). This result might be due to the fact that the condition of the plants in the screen house was more controlled than that outside the screen house. Inside the screen house, the plants were not exposed to abiotic stress, such as high temperatures, rain, and extreme temperature changes. This condition can affect plant resistance to *Begomovirus* infection. Simultaneous abiotic and biotic stresses can exert antagonistic, synergistic, or additive effects on plants, and these effects may lead to increased or decreased susceptibility to stresses. The great damage in the field with uncontrolled conditions can be attributed to secondary stress from a combination of two stressors exerting a negative or additive effect on plants (Asselbergh et al., 2008). Suleman et al. (2001) reported that drought stress in common beans infected with *Macrophomina phaseolina* leads to other symptoms. The same result was found when exogenous ABA was applied to detached tomato leaves; such application increases the susceptibility of wild type plants to *Botrytis cinerea* (Audenaert et al., 2002).

The results of the current study also showed that planting in the screen house led to a reduction in the population of whiteflies. Such outcome was deduced as the cause of the reduction in *Begomovirus* infection because this virus is only transmitted by whiteflies. This result was also reflected in the low scale of yellow leaf curling symptoms found in the plants in the screen house. Meanwhile, the plants outside the screen house showed diverse symptoms. This result may be due to the uncontrolled growth of whitefly infestation because *Begomovirus* can be carried by whitefly infestation from nearby weeds or crops that have been infected previously. Some wild plants (*Hyptis brevipes*, *Physalis floridana*, and *Crotalaria juncea*) and weeds (*Ageratum conyzoides*) are often found

around chili fields, and they are susceptible to *Begomovirus* infections (Sulandari et al., 2006).

As *Begomovirus* cannot be restrained directly, the most effective strategies are controlling the population of *Bemisia tabaci*. Whitefly, from the family Aleyrodidae and the Homoptera order, is a complex cryptic species of small insects with a piercing and sucking mouth type. These insects are distributed globally in tropical, subtropical, and low-climate regions (Wang et al., 2018). When infecting plants, whiteflies cause the shrinkage of plant nutrients as they spread the virus. Through their stylet, whiteflies suck the phloem liquid, thereby decreasing and even draining the nutrients in the leaves completely and causing the leaves to turn yellow. The reduction of leaf nutrition also causes a slope in plant photosynthesis activity while weakening the plants (Horowitz et al., 2011). Whitefly infestations affect plants in three stages of growth: nursery, flowering, and fruiting. Whiteflies in the nymph and imago stages suck the juice of plants through their mouth resting in a protected position in the rostrum. Whiteflies infect intercellular plant tissues and introduce fluid into the phloem, thereby inhibiting photosynthesis and affecting fruit conditions (Mohamed, 2012). Chemical control is not highly effective in preventing infestations because virus acquisition, as well as the development of virus resistance, requires a short time. Previous studies revealed the effects of insecticides on whiteflies after virus acquisition (Antignus, 2010). However, after a certain period of time, the effectiveness of insecticides decreases due to adaptations that cause resistance to insecticides; moreover, the relatively high toxicity of these insecticides for non-target organisms (including arthropods and humans) makes them ineffective in the efforts to control whitefly infestations (Mascarin et al., 2013; Wang et al., 2018).

Whiteflies only rely on their vision for navigation and orientation because their olfactory reaction is poor. They are sensitive to ultraviolet (UV) and the visible range of the electromagnetic spectrum. Numerous studies also reported that a disruption in UV vision might cause a disturbance in dispersal or orientation (Antignus, 2010). The screen of a screen house eliminates UV spectrum between 280 and 380 nm, thereby significantly diminishing the infestation of insects (including thrips, aphids, and whiteflies) on crop plants. The use of a screen has also been reported to reduce *Tomato yellow leaf curl virus* and *Cucumber yellowing stunting disorder virus* infections in tomato and cucumber (Kumar & Phoeling, 2006). Thus, using a screen house could hinder the entrance of whiteflies, which disrupt plant development. Although whiteflies were still found inside the screen house in the current study, they were fewer than those found outside the screen house.

Dramatic morphological and physiological changes in virus-infected plants result in the reduction of crop yield. Many previous reports revealed that virus infections can disrupt metabolic and

physiological processes, such as respiration, transpiration, and photosynthesis (Tajul et al., 2011). The results of the current study are in agreement with those of Khalil et al. (2014), who reported that *Begomovirus* infections in tomato cause the reduction of root length, number of plant leaves, shoot height, and the fresh and dry weight of shoots and roots of plants. The decrease in morphological quality and quantity might be related to the reduction of photosynthesis level due to the disruption of photosynthesis pigments. *Begomovirus* infections have been reported to cause mineral deficiency that increases the degradation/damage of the chloroplast thylakoid membrane (Khalil et al., 2014). Radwan et al. (2007) also revealed that *Zucchini yellow mosaic virus*-infected plants present decreased chlorophyll pigments. The disruption of chlorophyll pigments may be related to the production of plant defense hormones, such as salicylic acid (SA) and jasmonic acid (JA). JA and SA elicit the expressions of specific hormone-responsive genes that restrict invading pathogens (Spoel et al., 2007). Meanwhile, chloroplasts also play a role in innate immunity by restricting viral spread and systemic infections; they also serve as the site for defense hormone production. Chloroplasts undergo structural and functional damage as they become the main target of virus infections (Bhattacharyya & Chakraborty, 2018).

In the current work, the molecular detection of *Begomovirus* was conducted using coat protein genes. The coat protein gene sequence can be used to detect the presence of *Begomovirus* infection quickly and accurately with molecular characterization. CP gene sequences have conserved areas near the 5′ and 3′ ends and varied regions at the 5′ end along 200 nucleotides (Sinha et al., 2013). Thus, mutations in CP are associated with the emergence of *Begomovirus* variations (Subiastuti et al., 2019). Moreover, this sequencing has been used for the early identification of begomoviruses associated with cultivation plants in Mexico. However, the sequencing of the full DNA-A of *Begomovirus* needs to be carried out to achieve precise and accurate identification results (Hernandez-Zepeda et al., 2007). Previous studies also used this method for the quick identification of *Begomovirus* in melon (Subiastuti et al., 2019), tobacco (Widarta et al., 2017), tomato (Kusumaningrum et al., 2015), bitter melon (Tiwari et al., 2010), and mung bean (M. N. Islam et al., 2012).

Sample L5A was planted outside the screen house. Wild plants and weeds, as well as overlapping planting systems, were found near this study location. This condition facilitates the great prevalence of mixed infections (Subiastuti et al., 2019). The result herein also indicates that the sources of *Begomovirus* infection are nearby plantation crops or weeds that have been infected. *Ageratum* is one of the most common weeds found in fields near plantation crops, and they might be the source of the *Begomovirus* infection in this study. This phenomenon is in agreement with the results obtained by Shibuya et al. (2007) and Sakata et al. (2008), who found

that the Indonesia yellow vein disease in *Ageratum conyzoides* plants is caused by PepYLCIV. As reported by Mubin et al. (2009), weeds are reservoirs of *Begomovirus* that may be responsible for viral infections in crop plants. Weeds also facilitate virus recombination as they frequently harbor multiple viruses; this process results in new viruses/new strains. Various studies also reported *Begomovirus* infection in weeds. Graham et al. (2007) found that weeds of the genus *Sida* persistently harbored several begomoviruses originating from pseudo-recombination or molecular recombination; this finding led to the identification of *Sida micrantha mosaic virus*. Moreover, *Deinbollia mosaic virus*, a weed-infecting *Begomovirus*, has been reported to infect Solanaceae and Euphorbiaceae (Kyallo et al., 2017).

The L5 samples in the current work showed possible differences in PepYLCIV strains that infected the chili plants outside the screen house. This difference might be due to the condition of the plants in the screen house being more homogeneous than that outside the screen house. Further research is needed to obtain a full sequencing of the viral genome to complete the identification of the differences in the *Begomovirus* strains in the plants inside and outside the screen house and to determine potential polymorphism in both strains associated with DI or DS. The presence or absence of betasatellite and differences in DNA-A sequences might influence the severity of the disease in plants and host determination (Mansoor et al., 2003; Zubair et al., 2017).

CONCLUSION

Optical manipulation using a UV screen or a screen house was effective in reducing either *Begomovirus* infection or whitefly population. Understanding the source of infection will help to prevent the spread of infection. Molecular detection offers a fast method to detect the source of *Begomovirus* infection accurately while detecting its diversity. These strategies are expected to be a sustainable agriculture effort as they may reduce the use of insecticides and serve as alternatives to the development of resistant cultivars for controlling *Begomovirus* infections.

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Using Nematode Community to Evaluate Banana Soil Food Web in Mekargalih, Cianjur, West Java

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ABSTRACT

Soil biota is very diverse and contributes widely to ecosystem services that are important in the sustainable function of natural and managed ecosystems. Knowing the condition of the soil food web through the communities that inhabit it is necessary to assess the productivity of the soil. Nematode communities in the soil food web can be used as indicators because of their high abundance, and they inhabit various trophic levels, and participate in several important processes in the soil. The soil food web condition from three locations (Agr1, Agr2, Agr3) through the nematode functional index was evaluated using the maturity index (MI), the maturity index 2-5 (MI-25), the plant-parasitic index (PPI), the channel index (CI), the enrichment index (EI), the structure index (SI), and the basal index (BI). Nematode diversity was evaluated using Simpson's index of diversity, dominance, and evenness. The MI and MI2-5 scores indicated that Agr3 (3.81) had an undisturbed food web, while Agr2 (2.88 and 3.0) and Agr1 (2.5 and 2.51) were in a moderate condition with minor disturbances. Fauna profile analysis using SI and EI shows that Agr3 and Agr1 had an undisturbed soil food web, and Agr2 was in enriched conditions. CI results found that Agr1 and Agr3 had a fungal decomposition pathway while Agr2 had a bacterial decomposition pathway. This research showed that prospect of the nematode community to serve as a collection of biological indicator data in assessing soil or ecosystem health can be considered in further research.

Keywords: Agriculture, banana, food web, nematode

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INTRODUCTION

Soil is the foundation of terrestrial ecosystems, where the majority of the ecosystem services humans need come from. These ecosystem services are inherently dependent on soil health and the diversity of soil food web biota. They play a role in nutrient cycling, mineralization, and decomposition. The agricultural practice affects the soil food web structure and services providing by the soil (de Vries et al., 2013). Banana is one of the leading fruit commodities in West Java and contributes about 15.5% of national production (Statistics Indonesia [BPS], 2019). In West Java, Cianjur Regency is the largest banana producer with 1.12 million tons in the last five years (BPS, 2019). It is important to regularly evaluate soil food webs for sustainable crop yields and soil health (Kibblewhite et al., 2008).

Nematodes can be used as indicators of the soil food web because they meet several conditions as a good indicator: large abundance, varying trophic levels, and diverse life strategies (Brussaard et al., 2004). Nematodes also have several advantages as indicators: nematodes have a short generation period that allows them to respond quickly to food availability and environmental disturbance (Bongers, 1990; Wasilewska, 1989). Nematodes trophic levels are also useful for determining ecosystem function, and they are easily identified and low in cost (Neher et al., 1995). The nematode community structure index also reflects the process of soil succession (Neher & Campbell, 1994) and can be used as a bioindicator in monitoring the condition of agricultural soils (Bongers & Ferris, 1999; Wasilewska, 1989; V. R. Ferris & Ferris, 1974).

To assess soil food webs through the nematode community several indexes developed using the colonizer-persister scale (c-p scale), such as the maturity index (MI) for free-living nematodes and the plant-parasitic index (PPI) for herbivorous nematodes (Bongers, 1990). The nematode community can be used to analyze food web conditions and describe ecosystem succession levels based on groups of nematode feeding habits. Nematode communities were accessed using an ecosystem functional index consisting of enrichment index (EI), basal index (BI), channel index (CI), and structural index (SI) (H. Ferris et al., 2001). Nematodes have the potential as bioindicators for assessing disturbance from banana system practices and evaluating soil health.

Research on nematodes to evaluate soil and environmental health in Indonesia is still limited. Nematode studies in Indonesia focus on plant-parasitic nematodes (Budiman et al., 2020; Djiwanti, 2019; Handayani et al., 2020; Kurniawati et al., 2020; Lisnawita et al., 2012; Mirsam et al., 2020; Mutala'liah et al., 2018), parasites in vertebrates (Ahmad & Tiffarent, 2020; Baihaqi et al., 2019; Khoirani et al., 2020; Sakaguchi et al., 2019), and nematode ecology (Krashevska et al., 2019). Therefore, this research needs to be carried out considering the great benefits and advantages of nematodes as bioindicators and the limited research on

nematode ecology in Indonesia. This study aimed to evaluate banana soil food webs using nematode communities.

MATERIALS AND METHODS

Experimental Design

The experimental design in this study is quantitative observation. The nematode functional index was used to analyze community data taken from three locations (Agr1, Agr2, and Agr3). The results of the analysis were described according to the interpretation of each index and did not use any statistical analysis.

Site Description

Soil sampling was in Mekargalih Village, Cianjur Regency, West Java, Indonesia because it is the largest banana producer in West Java (BPS, 2019). Three banana systems were used for soil sampling with all location coordinates recorded using the Global Positioning System (GPS) from Google Maps and converted to sexagesimal using Microsoft Excel. Agr2 (6°41'37" S, 107°12'5" E) is the largest banana system with 481 m^2, the second largest is Agr3 (6°41'31" S, 107°12'9" E) with 433 m^2, and the smallest is Agr1 (6°41'34" S, 107°12'6" E) with 412 m^2. Soils from all locations have similar physicochemical properties: high humidity, clay type, dark brown color, acidic, moderate-to-high soil organic carbon (SOC), and high cation exchange capacity (CEC) (Table 1).

Banana plants at each location had different ages; two months in Agr1, 15 months in Agr2, and 14 months in Agr3. At the beginning of planting, soil preparation was given, such as manure application, lime application, fungicide, and ridge tillage with soil hoeing. Agr1 and Agr2 had no other vegetation besides banana plants, while Agr3 was full of weeds.

Table 1

Soil physicochemical properties of all locations

	Moisture (%)	Sand (%)	Silt (%)	Clay (%)	Texture	Color	pH (H_2O)	SOC (%)	CEC (cmol(+)/ kg)
Agr1	78	7	26	67	Clay	Dark brown	5.57	1.50	33.98
Agr2	70	6	40	54	Silty clay	Dark brown	5.48	2.45	33.98
Agr3	70	5	27	68	Clay	Dark brown	5.57	1.77	38.02

Note. SOC = Soil organic carbon; CEC = Cation exchange capacity

Soil Sampling and Nematodes Identification. A total of 3 composite samples were taken in January 2020 from 3 locations (Agr1, Agr2, and Agr3). Each composite sample consisted of 10 sub-samples taken using a zig-zag pattern along the transect (Coyne et al., 2014). There were 10 sub-samples taken because the land area is less than 500 m^2 (Celleti & Potter, 2016). Sub-samples were taken at 15-20 cm depths (Hooper et al., 2005) using soil augers (4 cm diameter) (Van Bezooijen, 2006) and stirred to become composite samples (Hooper *et al*., 2005). Samples were placed in labeled plastic bags and transported to the laboratory in a cooler box. Nematodes were isolated from 100 g of soil using a modified Baermann funnel method (Van Bezooijen, 2006). Nematodes were transferred to the top of the slide using a micropipette and observed using high magnification under a microscope (Nikon *ECLIPSE* E100LED MV R).

Nematodes were identified based on morphological characteristics (*e.g.* stoma shape, feeding apparatus, pharyngeal shape, genital branch, and tail shape) down to the family level using several keys for nematode identification. Order of Rhabditida: Andrássy (1983); Nguyen (2006, 2009); Scholze and Sudhaus (2011). Order of Dorylaimida: Jairajpuri and Ahmad (1992); Peña-Santiago (2006, 2014a); Vinciguerra (2006). Order of Mononchida: Peña-Santiago (2014b); Zulini and Peneva (2006). Order of Tylenchida: Siddiqi (2000); Subbotin (2014).

Nematodes Community Analyses

Nematodes were identified to the family level because they have the same anatomical and physiological attributes and feeding habits that are useful for fauna analysis (H. Ferris et al., 2001). The diversity index of the nematode community at each location was calculated using Simpson's index of diversity, dominance, and evenness. Simpson (1949) gives a formula for dominance and diversity index as:

$$\lambda = \sum \frac{n_i[n_i - 1]}{N[N - 1]} \quad [1]$$

$$D = 1 - \lambda \quad [2]$$

Pielou (1969) provide a formula for the evenness index derived from Simpson's dominance as:

$$E = \sum_{i=1}^{n} \frac{v_i \cdot f_i}{N} \quad [3]$$

where λ is Simpson's dominance, D is Simpson's diversity, n_i is the number of individuals in family *i*, N is the total individuals in the community, and S is the number of families in the community. Interpretation refers to the guidelines for interpreting the Simpson diversity index scores by Guajardo (2015) (Table 2).

The nematode family is classified into functional groups and colonizer-persister scales (c-p). Colonizer-persister (c-p) is a scale assigned from 1-5 to the nematode family based on r and K characteristics (H. Ferris et al., 2001). The characteristics of the r are tolerant to disturbance, as decomposers, high metabolic activity, high fecundity, and a short life cycle. K group is sensitive and

Table 2
Guidelines for interpreting Simpson diversity index scores

Simpson score	Interpretation
0.0	Absence of diversity (homogeneity)
0.01 – 0.4	A low degree of diversity/heterogeneity
0.41 – 0.60	A moderate degree of diversity/heterogeneity
0.61 – 0.80	A moderately high degree of diversity/heterogeneity
0.81 – 0.99	A high degree of diversity/heterogeneity
1.0	Absolute (perfect) diversity/heterogeneity

tends to be in undisturbed conditions, large nematodes, long life cycle, low fecundity, and consists of carnivores and omnivores (Bongers, 1990; H. Ferris & Bongers, 2006; H. Ferris et al., 2001).

The weighted average of nematode communities was calculated using the c-p scale for each family or the so-called maturity index (MI) and plant parasite index (PPI) (H. Ferris et al., 2001). MI scores varied from 1 in the highly enriched state to 5 in the undisturbed condition. H. Ferris et al. (2001) provide a general formula for calculating the index in MI families as:

$$XI = \sum_{i=1}^{n} \frac{v_i \cdot f_i}{N} \quad [4]$$

where XI is the index of interest (MI for free-living and PPI for plant-parasitic nematode), v_i is the colonizer-persister (c-p) value assigned to taxon *i,* and f_i is the number of nematodes in each of the *f* taxa that meet the criteria of the index. Interpretation of MI values based on the classification is given by Moreno et al. (2011).

Analysis of soil food webs has been measured based on the presence and abundance of nematode functional groups (feeding habits or trophic level). The determination of the functional groups refers to the description by Yeates et al. (1993), based on the principle that the presence and abundance of functional groups in an ecosystem is a consequence of environmental change (H. Ferris et al., 2001). H. Ferris et al. (2001) then classified the food web into 3 conditions: *b* (basal), *e* (enriched), and *s* (structured), and in their application they are expressed as the value of the fauna component (*b*, *e*, and *s*). *b* is the value of the basal group (Ba_2 and Fu_2) in the community, and it can be calculated as:

$$b = (Ba_2 + Fu_2) \cdot W_2 \quad [5]$$

Ba_2 is a group of bacterivore c-p2 and Fu_2 is a group of c-p2 fungivore nematodes. *e* is the value of the opportunist nematode group (Ba_1 and Fu_2) in the community, so it can be expressed in:

$$e = (Ba_1 \cdot W_1) + (Fu_2 + W_2) \quad [6]$$

Ba_1 is the number of individuals of the bacterivore c-p1 group and Fu_2 is the number of individuals of the fungivore c-p2 group. *s* is the value of all functional

groups present in the community, and it can be calculated as:

$$s = (Ba_n \cdot W_n) + (Fu_n \cdot W_n) + (Ca_n \cdot W_n) + (Om_n \cdot W_n) \quad [7]$$

In *s*, all the c-p of bacterivores (Ba_n), all the c-p of fungivores (Fu_n), all the c-p of carnivore (Ca_n), and all the c-p of omnivore (Ca_n) in the community were counted. W_n is the weight or constant of the functional group W at c-p *n*, H. Ferris et al. (2001) assigned weights as follows: 3.2 for W_1; 0.8 for W_2; 1.8 for W_3; 3.2 for W_4; 5 for W_5 (Figure 1).

Based on the food web classification, H. Ferris et al. (2001) formulated the ecosystem functional index. This index consists of the structure index (SI), the enrichment index (EI), the basal index (BI), and the channel index (CI). SI provides information on the complexity of nematode interactions between levels in the food web. SI can be stated in:

$$SI = \frac{s}{s+b} \cdot 100 \quad [8]$$

Since all nematode functional groups in the community were included in the calculation, the SI values can be used as an indicator of a good soil food web (scale 0 degraded - 100 highly structured). EI provides information regarding the activities of opportunist groups (Ba_1 and Fu_2) that react to the availability of food sources in the soil, so that it can be expressed in:

$$EI = \frac{e}{e+b} \cdot 100 \quad [9]$$

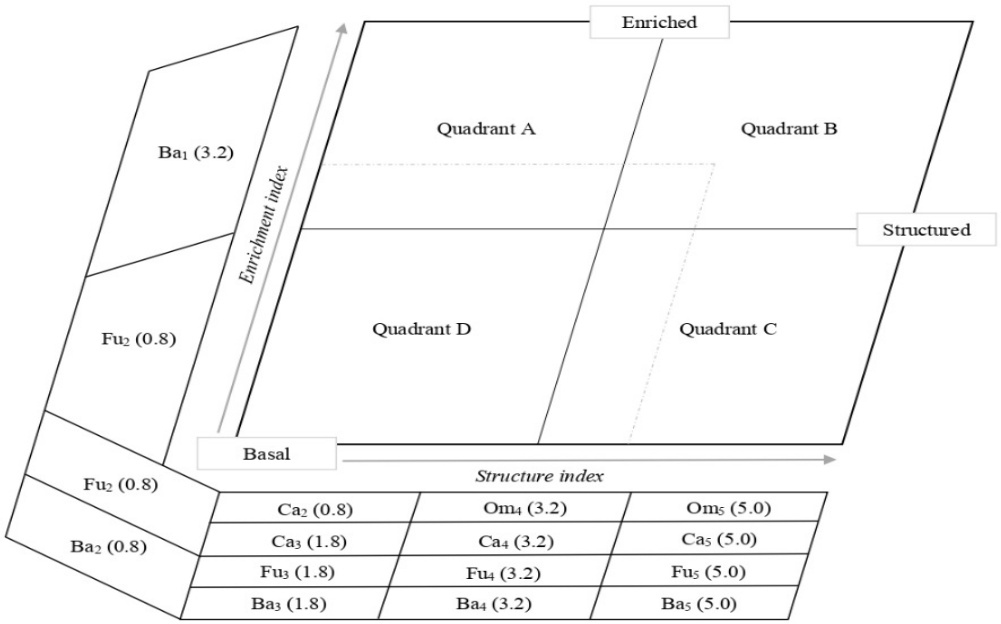

Figure 1. Fauna profile analysis of soil food webs. Divide the food web conditions into 4 quadrants and 3 criterias. It also shows the weight of each c-p of the nematode functional group (H. Ferris et al., 2001)

The EI value can be used to evaluate the availability of resources in the food web. A high EI can indicate that the soil is being enriched (e.g. manure application, the addition of plant litter, or natural disaster). The EI value is on a scale of 0-100 where 0 indicates a depleted resource and is 100 heavy enrichment. BI is an indicator of the level of disturbance in the soil food web, so that it can be expressed in:

$$BI = \frac{b}{e+s+b} \cdot 100 \quad [10]$$

On a scale of 0-100 on BI, a value of 0 indicates a highly degraded soil food web and a value of 100 indicates an undisturbed soil food web. CI is calculated as the proportion of fungal-feeding nematodes compared to enrichment-opportunistic bacterial-feeders nematodes. It provides information regarding the decomposer pathway in the soil food web and can be expressed in:

$$CI = \frac{Fu_2 \cdot W_2}{Ba_1 \cdot W_1 + Fu_2 \cdot W_2} \cdot 100 \quad [11]$$

If the CI value <50 that indicates the soil food web has a bacterial decomposition pathway, if the CI value >50 indicates that the soil food web has a fungal decomposition pathway, and the CI value of 50 indicates that the soil food web has a balanced decomposition pathway, where Ba, Fu, Ca, and Om are the abundance of bacterivorous, fungivorous, carnivorous, and omnivorous nematodes; n is the c-p value for the nematode families; W is the weight of each c-p of functional group: c-p1 3.2; c-p2 0.8; c-p3 1.8; c-p4 3.2; c-p5 5 (Figure 1).

Its use to interpret food web conditions, the ecosystem functional index is not only used as a single standing index. Berkelmans et al. (2003) combined SI and BI to describe the effects of agricultural land management on soil food webs through nematode communities. Then H. Ferris et al. (2001) developed a fauna profile analysis based on a combination of SI and EI, which was transformed into a Cartesian diagram and divided into four quadrants and three conditions (Figure 1). Each quadrant represents different food web conditions.

RESULTS AND DISCUSSION

Diversity of the Nematode Families

Fifteen families of nematodes were identified and classified into their functional groups (Table 3): five bacterivores, one fungivore, four herbivores, three omnivores, and two carnivores. From the diversity index, the communities in Agr1 have high diversity, while Agr2 and Agr3 in moderate. Richness (Hill's H_0) and evenness (Jost, 2010) affect the diversity index value. Agr1 has the highest diversity because it is richer and has a more even distribution of abundance. Two families dominate the Agr2 community (Hoplolaimidae and Meloidogynidae) and three families dominate the Agr3 (Hoplolaimidae, Heteroderidae, and Meloidogynidae). The low dominance index value indicates that there are no taxa that dominate the abundance and on the even distribution in a community.

Table 3
Abundance of nematode functional groups in each location

Family	c-p	Abundance			Functional groups
		Agr1	Agr2	Agr3	
Rhabditidae	1	1	1	-	Bacterivorous
Cephalobidae	2	50	5	1	Bacterivorous
Rhabditonematidae	2	9	-	1	Bacterivorous
Ostellidae	2	1	-	-	Bacterivorous
Aphelenchidae	3	14	3	1	Fungivorous
Teratocephalidae	3	2	-	-	Bacterivorous
Hoplolaimidae	3	26	33	52	Herbivorous
Meloidogynidae	3	16	26	13	Herbivorous
Pratylenchidae	3	-	5	4	Herbivorous
Heteroderidae	3	-	7	12	Herbivorous
Dorylaimidae	4	9	1	5	Omnivorous
Nordiidae	4	12	5	9	Omnivorous
Qudsinematidae	4	2	1	-	Omnivorous
Mononchidae	4	-	1	2	Carnivorous
Aporcelaimidae	5	-	-	2	Carnivorous

Note. c-p = Colonizer-persister

High-dimensional ecological data are summarized using a diversity index for easy understanding and comparison (Daly et al., 2018). The summary may also result in missing some important information from the community. Agr1 has a good diversity index value, but the function of the taxa present is unknown. Therefore, a functional index is needed to describe each taxa function that is present in the community.

Soil Food Web Condition

All locations are categorized as semi-conventional with manure, lime, and fungicides applications, using ridge tillage, no crop rotation, and monoculture. The abundance and diversity of nematodes in semi-conventional farming are smaller than in organic agriculture and natural ecosystems. Besides that, the families present were not much different. Apart from being limited by environmental conditions and soil physicochemical properties, nematode communities are also greatly influenced by the plants growing on the soil (Freckman & Caswell, 1985).

Manure application increases the abundance and richness of soil nematodes (X. Liu et al., 2020). Manure increased the abundance of nematodes in Agr1 by 36.46%

compared to Agr2 and 28.67% against Agr3. The abundance of the opportunist group decreased from day 21 (Ettema & Bongers, 1993) to 77 (Sholeha et al., 2017) days after manure application due to reduced resources. The meta-analysis conducted by T. Liu et al. (2016) stated that manure increased nematode abundance by 37%.

Ridge tillage is part of conventional tillage (CT) and affects soil nematode communities (Okada & Harada, 2007). This study shows that Agr1 (CT) has a higher diversity index, evenness index, PPI, and PPI/MI compared to no-tillage (NT) Agr2 and Agr3. CT decreases MI, MI2-5, SI, and increases PPI. The abundance of bacterivores (44.06%) was higher and lower herbivores (29.37%) in Agr1 compared to Agr2 (6.81% and 81.61%) and Agr3 (1.96% and 79.41%). Research from Lenz and Eisenbeis (2000) also shows similar results. Agricultural practices such as CT cause changes in soil physical properties that affect the soil nematode community structure (Wardle, 1995).

This study showed the highest abundance of c-p2 Cephalobidae (Agr1 34.9%) followed by the lowest EI coming from soil treated with fungicides (Agr1). Fungicide treatment was also associated with a decrease in predatory (omnivores and carnivores) and the low abundance of the bacterivore group (Ortiz et al., 2016; Yardim & Edwards, 1998). Predators on Agr1 (16.78%) were lower than Agr3 (17.65%) but higher than Agr2 (9.19%). The c-p2 abundance of Cephalobidae is hypothesized more affected by enrichment than the fungicide treatment. The nematode community had already recovered from the fungicide due to the small amount applied in the early of the plantation. Fungicides have less impact on the soil community than insecticides and herbicides (Foissner, 1997) and enrichment in the soil is a disturbance that can cause retrogression in the nematode succession process (Neher, 1999).

The lime treatment has no effect on the soil nematode community (Varga et al., 2019) because nematodes are more affected by soil pH. Damage caused by herbivorous nematodes has increased in monoculture agriculture (Quénéhervé et al., 2011). Crop rotation can improve soil quality, such as soil community structure, organic matter, and moisture (Ponge et al., 2013). A long-term crop rotation on banana farming was able to increase MI, SI, EI, diversity index, and led to a highly structured food web reported by Zhong et al. (2015). The fallow effect is also needed to restore the nematode community structure and reduce the herbivorous nematode population (Masse et al., 2002; Wang et al., 2004).

Vegetation type (Cesarz et al., 2013) and soil properties determine the shape of nematode communities as they respond to changes in the environment (Nielsen et al., 2014). Bacterivore and fungivore groups are low in soil with acidic conditions. However, the higher abundance of Agr1 resulted from manure application. Research by Renčo et al. (2020) also got the same result. The abundance of the bacterivore, fungivore, and herbivore groups has a positive correlation with soil moisture. Omnivore and carnivore

abundance has a negative correlation with soil moisture (Nielsen et al., 2014; Treonis et al., 2019). The abundance of the bacterivore and fungivore groups in Agr1 was higher than Agr2 and Agr3 (Table 3). Meanwhile, the lowest herbivore abundance in Agr1 was due to single vegetation (banana).

Soil organic matter (SOM) content correlated positively with the bacterivore and fungivore groups due to microbial activity (Treonis et al., 2019). Otherwise, Barros et al. (2017) reported that SOM negatively correlated with herbivore groups. However, Agr1 had the highest abundance of bacterivore and fungivore groups. Agr2 and Agr3 with higher SOM content had a very low richness of bacterivores and fungivores. Sandy soil textures are preferred for nematodes and have a greater abundance than clay soils. Larger pores in sand texture are easier for aeration and the spatial distribution of the nematodes (Kim et al., 2017). The c-p scale assessment was applied to the identified nematode families. Based on the criteria of r and K (H. Ferris et al., 2001), c-p1-2 nematodes are sensitive to enrichment and the most resistant to environmental disturbances. Meanwhile, the sensitive group to environmental disturbances and changes belongs to c-p4-5 nematodes (Bongers & Bongers, 1998). This concept makes it possible to determine the disturbance level and the ecosystem succession rate using MI. Increasing MI value indicates succession and decreasing MI value indicates retrogression.

In measuring ecosystem disturbance, Korthals et al. (1996) modified the MI by leaving the c-p1 nematode from the MI calculation (which can cause bias) known as MI2-5. The PPI is used to measure the average weight of family frequencies based on the c-p scale for herbivorous or plant-parasitic nematodes. PPI is compared with the MI value which is called the PPI/MI ratio. Enrichment in the soil can cause the ratio value to decrease due to decreasing MI value and *vice versa* (Bongers & Korthals, 1995). The evaluation of well-controlled herbivore nematode populations by carnivorous and omnivorous nematodes is suggested to use the PPI/MI score.

Result of the MI (Table 4), Agr3 has a high MI score with succession already at the structured level. The structured level had a higher abundance of the c-p4-5 group (17.65%) than the c-p1-2 group (2.94%). Agr2 has a good MI score with succession at the mature level. The good level succession has an abundance of the c-p4-5 group (9.09%) relatively balanced than the c-p1-2 group (10.23%). Agr1 has a moderate MI score and succession also at moderate levels. For moderate or lower succession level (disturbed and degraded), the community composition is dominated by the c-p1-2 group (51.75%) than the c-p4-5 group (16.78%).

In interpreting the MI score, the cause of the decrease in the MI needs to be known for a more comprehensive understanding. Approximately five-six weeks before the soil sample taken from Agr1 manure is applied. Less than a week after manure application, the abundance of opportunist groups (Ba1 and Fu2) (Bongers & Bongers, 1998) increased due to high soil microbial activity. After three weeks, basal groups

Table 4
Diversity and ecological functional index

	Agr1	Agr2	Agr3
Diversity index	0.81	0.76	0.70
Dominance index	0.19	0.24	0.30
Evenness index	0.45	0.38	0.31
Hill's H_0	12	11	11
Maturity index	2.5	2.88	3.81
Maturity index 2-5	2.51	3	3.81
Plant-parasitic index	3	3	2.95
PPI/MI	1.2	1.04	0.77
Structure index	70.95	84.62	96.36
Enrichment index	23.32	52.24	42.86
Channel index	77.78	42.86	100
Basal index	26.69	13.17	3.54

(Ba2 and Fu2) such as Cephalobidae (Bongers et al., 1991) replace the population of opportunist groups due to decreased resources of the manure (Ettema & Bongers, 1993). Thus, the abundance of Cephalobidae on Agr1 was not due to disturbances in the soil food web but a succession process after manure application. The presence of c-p4-5 nematode on Agr1 also indicates that the fungicide was applied in low doses. According to Briar et al. (2012), the omnivore and carnivore c-p4-5 groups did not appear in chemically treated soils before disturbance levels could be reduced or eliminated.

Basal groups (c-p2 Cephalobidae) can survive in disturbed conditions because they can enter the anhydrobiosis stage and dauer larvae if environmental conditions are not favorable. The life cycle resume once the conditions are favorable (McSorley, 2003). MI2-5 is used to measure the level of disturbance based on the c-p scale of families present in the community. From the MI2-5 score, Agr1 and Agr2 are soil food webs with minor disturbances and Agr3 with stable conditions.

The PPI/MI shows that Agr3 has a score of <1 (Table 4), while Agr1 and Agr2 have a score >1. Based on their role in the ecosystem, the presence of herbivores provides a different interpretation from free-living. A single herbivore individual remains a threat to plant health and crop yields. However, eliminating herbivorous groups is impossible. Thus, the PPI/MI score can be used to assess the risk of herbivore groups towards agriculture. If the score is <1, the herbivore population is under control and if the score is >1, it is necessary to

take precautions to reduce the herbivorous nematode population.

Agr1 and Agr3 have a fungal pathway, and Agr2 has a bacterial pathway but is close to balance. The fungal pathway shows two things: the soil litter is dominated by lignin and cellulose; slow decomposition pathway (Frouz et al., 2013); succession is moving from an early stage as it changes from a bacterial pathway to a fungal pathway (Wardle et al., 1995). The bacterial pathway also shows two things: the litter in the soil is dominated by a matter of C: N ratio below 30:1; fast decomposition pathway; and shows that the food web is still at an early stage of succession (Frouz et al., 2013).

Berkelmans et al. (2003) used SI and BI to examine the impact of the agroecosystem on nematode communities. In this study (Figure 2), Agr3 has an excellent soil food web condition with SI 96.36% and BI 3.54%. Agr2 has a good food web condition with SI 84.62% and BI 13.17%. Agr1 has moderate soil food web conditions with SI 70.95% and BI 26.69%. Agr3 has the most complex and undisturbed food web conditions shown in its high SI score. The presence of families of nematodes with various tropic levels influenced this score: bacterivore Cephalobidae, fungivore Aphelenchidae, carnivore Mononchidae, and omnivore Aporcelaimidae. From the BI score, this study shows that Agr3 has the best and undisturbed food web, followed by Agr2, and Agr1. BI is influenced by the abundance of c-p2 nematodes in the community and is most abundant in Agr1 (Cephalobidae).

The enrichment index (EI) is an indicator of resource availability on the food web. Enrichment conditions in the soil food web can occur due to additional external resources, disturbance, environmental shifts, and the death of organisms (Odum, 1985). H. Ferris and Bongers (2006) also concluded that the addition of any suitable material as a substrate for bacteria and soil fungi would trigger an increase in the number of c-p1 nematodes and some c-p2 nematodes. Agr2 has the highest resources (EI 52.24) and is classified as enriched soil (EI >50) (H. Ferris et al., 2001) while Agr3 (42.86) and Agr1 (23.32) have moderate resources (EI <50). The abandoned agricultural land can substantially increase soil C and N storage (Compton & Boone 2000), due to an increase in plant diversity (Lange et al., 2015). Resources on Agr2 and Agr3 come from the litter of various growing plants (grass, shrubs, and banana leaves). Meanwhile, in Agr1 the resources only depend on manure because there are only banana plants.

Analysis of the fauna profile (Figure 3) shows that Agr1 and Agr3 belong to the C quadrant and Agr2 belongs to the B quadrant. Based on H. Ferris et al. (2001) for the interpretation of fauna profiles, quadrant C is a food web with the following criteria: undisturbed food web, moderate enrichment, fungal decomposition pathways, moderate to high C:N ratio content, and structured. Quadrant B has the following criteria: low disturbance, obtaining N enrichment, balanced decomposition pathway, low content of C:N ratio, and maturity.

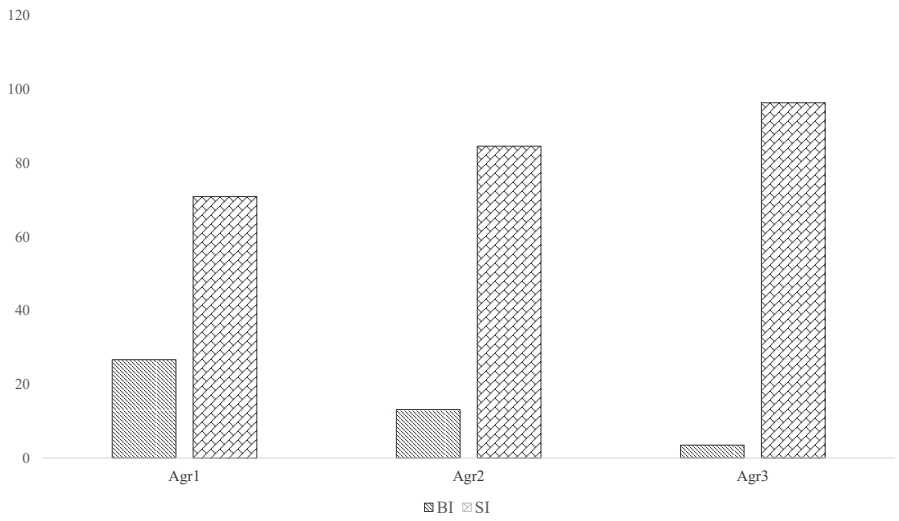

Figure 2. Comparison of SI and BI from all locations

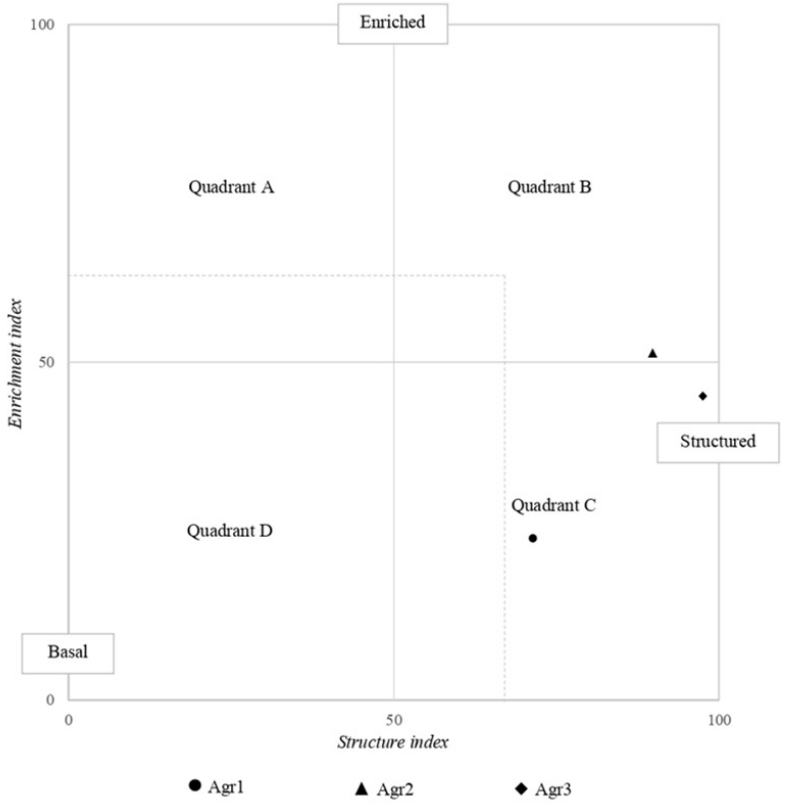

Figure 3. The fauna profile analysis showed that Agr1 and Agr3 belong to the C quadrant and Agr2 to the B quadrant

CONCLUSION

In this work, the agricultural practices applied to the soil form nematode communities. Tillage and manure increase the group of opportunists and decrease the group of omnivores and carnivores. Soil moisture influences the abundance of bacteria and fungivores. Agr3 has a very complex interaction between trophic, the lowest disturbance level, and structured level succession. Agr2 has complex trophic interactions, low disturbance level, and a mature level succession. Agr1 has moderate interactions between trophic, the highest disturbance level among other locations, and a moderate level succession.

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Foraging Behaviour of *Heterotrigona itama* (Apidae: Meliponini) in Residential Areas

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ABSTRACT

This study aims to investigate the foraging behaviour of *Heterotrigona itama* in exploiting food resources at a residential area, and the viability of this species to adapt to urban microclimatic conditions. *Heterotrigona itama* prefers to forage at areas closer to their nesting site, where diverse food sources are found. The marked bees of *H. itama* prefer to forage on various resources available at a 500-metre radius from the house yard. The obtained results indicate that the active foraging pattern of *H. itama* is negatively correlated to the time phases of a day ($p < 0.05$). This phenomenon was contributed by the three peaks of foraging hours, which reached a peak in the early morning (6:30 to 8:00 a.m.), moderately peaked towards the evening (2:30 to 3:30 p.m.), and was greatest towards the afternoon (10:30 a.m. to 12:00 p.m.). The ambient temperature and relative humidity were not the primary factors influencing the average number of foragers exiting from and returning to the hives (temperature, $p > 0.05$; and humidity, $p > 0.05$). There was a difference between the varieties of content resources collected by the bees ($p < 0.05$). The nectar or water sources was the highest material (51.39%) that was brought back to the hive by foragers, followed by resin (34.73%) and pollen (13.87%). There was a significant difference in foraging time

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phases by returning foragers for collecting resin ($p < 0.05$) and nectar or water ($p < 0.02$), but there was no significant difference in foraging time phases found for pollen ($p > 0.05$). The results showed that *H. itama* is able to withstand urban microclimate conditions, and successfully incorporated pollen, nectar or water, and resin obtained from floral and non-floral resources into their diet.

Keywords: Abiotic factors, foraging behaviour, *Heterotrigona itama*, residential area, stingless bees

INTRODUCTION

Compared to honeybees, stingless bees are relatively easier to handle, less aggressive as they do not have a functional stinger, and are efficient in foraging ability, thus making them ideal to be reared in residential areas. Nowadays, urban beekeeping in residential areas demonstrates a rise in popularity in Malaysia (Basari et al., 2018). Beehives require minimal land, and can be placed almost anywhere, including house yards and orchards. However, the foraging activity of stingless bees in an urban environment remains unclear. In the State of Sabah, beekeeping with *Heterotrigona itama* has induced a massive growth in interest, particularly on the west coast where many beekeepers want to keep colonies of bees in residential areas as a hobby, income opportunity, or for their own source of authentic honey. The direct contributions of stingless beekeeping include the value of the output produced such as honey, beebread, and one of essential ingredients in cosmetics and medicine, known as propolis (Yaacob et al., 2018). The indirect but significant contribution of beekeeping is through floral pollination in agricultural and natural environments (Heard, 1999; Roubik, 2006). The flora of the residential area wherein the beehives are situated is highly benefitted due to pollination activities by the bees (Roubik & Buchmann, 1984).

Heterotrigona itama is a common stingless bee species found in Southeast Asia, and is among the most popular species in meliponiculture (Heard, 1999; Mustafa et al., 2018). Due to its popularity, it has been selected as a model organism in this study. This species can be easily identified from other commonly encountered species with similar colouration, as it has a wider and longer body (Samsudin et al., 2018). For example, the main feature of the genus *Tetrigona* spp. (e.g. *Tetrigona apicalis* and *Tetrigona binghami*) is its large white-tipped wings, while the *Tetragonula* spp. (*Tetragonula laeviceps* and *Tetragonula fuscobalteata*) is much smaller than other genera of stingless bees. Thus far, the effects of climatic factors (e.g. temperature and relative humidity) towards the flight activity of *H. itama* in urban areas remains poorly understood. Temperature and relative humidity are vital environmental factors that may affect the foraging activity of stingless bees (Hilário et al., 2000). The climatic factors have been reported to influence the flight activity of *Tetragonula carbonaria* (Heard, 1999). Keppner and Jarau (2016) also found that the foraging activity of *Partamona*

orizabaensis escalated in weather conditions such as colder temperatures and increased relative humidity, as well as during rainfall. The climatic factors aid honeybees and bumblebees in orientating and navigating their environments, as successful orientation is vital for foragers to return to their nest after foraging (Moore & Rankin, 1983; Stelzer et al., 2010).

Previous studies have shown that eusocial bees exhibit high preference in exploiting material sources for the survival of their colonies (Moore & Rankin, 1983; Nagamitsu & Inoue, 2002; Stelzer et al., 2010). Thus, urbanisation may affect the bees' flight activities due to changes in the availability of local resources. However, data remains limited. The bees require floral resources to survive, and therefore, private gardens, parks, and wild floral habitats within urban landscapes may support food and nesting resources (Ropars et al., 2019; Udy et al., 2020). Although urban beekeeping is growing in popularity in Sabah, the bees' foraging activity in residential areas has been poorly investigated. Therefore, this study aims to investigate the foraging behaviour of *H. itama* in exploiting food resources in residential areas, and the viability of species to adapt to urban microclimatic conditions.

MATERIALS AND METHODS

Study Site

This study was conducted in one of the residential areas located in Lot 19, Taman Sejati Ujana, Sandakan, Sabah, which has side a yard size of 500 square feet (Figure 1). The surrounding areas of Taman Sejati Ujana comprise many old and new housing project developments (Figure 1). The type of floral landscape is described in Table 1.

Beehives Placement and Hives Management in House Yard

Fourteen colonies of *H. itama* were reared for 22 months (January 2018 to October 2019). All methods were standardised for all studied beehives, and the layout and orientation of the stingless beehives within the house yard are shown in Figure 2. These beehives comprise bee colonies with weak and strong colonials' nests development. Strong bee colonies can be distinguished by beehives having active foraging traffic and well-formed nest entrance tubes, with many bee guards defending the nest. All the beehives were placed on wood stands at a height of 50 to 60 cm above the ground.

All beehives were placed under a tree canopy or in the shade. Beehives placed under open light were covered with a plywood roof lined with rubber mats or aluminium. The roof was designed to protect the bee colony from the sun and rain (Figure 3).

Sites Foraging Preferences of *Heterotrigona itama* within a Residential Area

The insects visiting the vegetation were observed for 30 minutes in each of the sites of A, B, C, D, E, F, G, and around residential areas (Site H) (Figure 1), from 8:00 a.m. to 5:00 p.m., twice a week, for five months. Two observers were trained to spot and

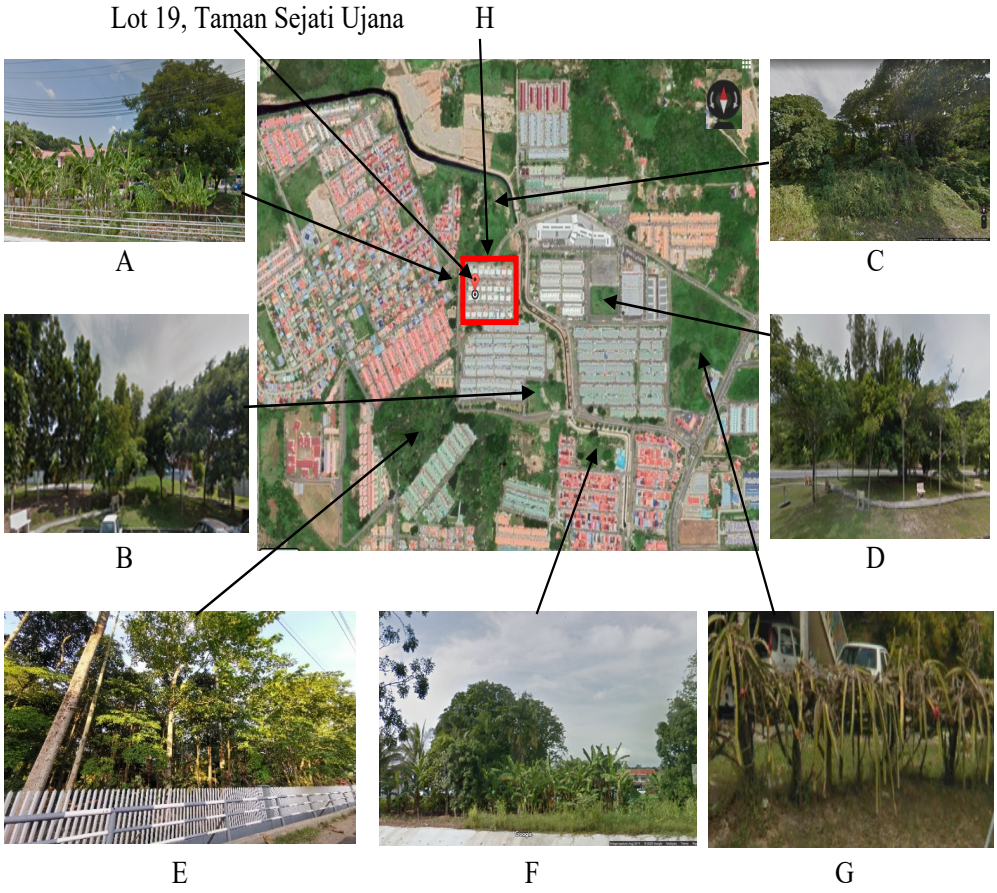

Figure 1. Location of bee colonies in Lot 19, Taman Sejati Ujana, Sandakan, Sabah and the distribution of floral areas

Table 1

Categories of floral landscape observed within and outside the residential area

Study sites	Floral categories	Floral types	Straight line distance measured from study site (m)
A	Private agriculture area 1	Banana, mango, papaya, shrubs, leafy vegetables, tapioca, sugarcane, corn, citrus, coconut, legumes, *Tunera* sp., *Passiflora* sp., and fruity vegetables	50
B	Park 1	Park (Landscape trees)	60

Table 1 *(Continued)*

Study sites	Floral categories	Floral types	Straight line distance measured from study site (m)
C	Unmanaged landscape	*Acacia* sp., *Macaranga* sp., *Melastoma malabathricum*, *Mimosa pudica*, *Ageratum* sp., *Passiflora* sp., *Imperata cylindrica*, *Mikania micrantha*, and unknown wild plants	95
D	Park 2	Park (Landscape trees)	90
E	Natural forest	Class IV Forest Reserve Timber trees, exotic and indigenous trees, shrubs and bamboo	350
F	Private agriculture area 2	Banana, mango, shrubs, vegetables, tapioca, coconut and seasonal flowering trees	500
G	Private garden	Dragon fruit	1000
H	Taman Sejati Ujana	Banana, mango, papaya, shrubs, leafy vegetables, citrus, guava, mulberry, chilli, tapioca, coconut, coral vines, *Tunera* sp., *Ixora* sp., and seasonal flowering trees	0 – 40

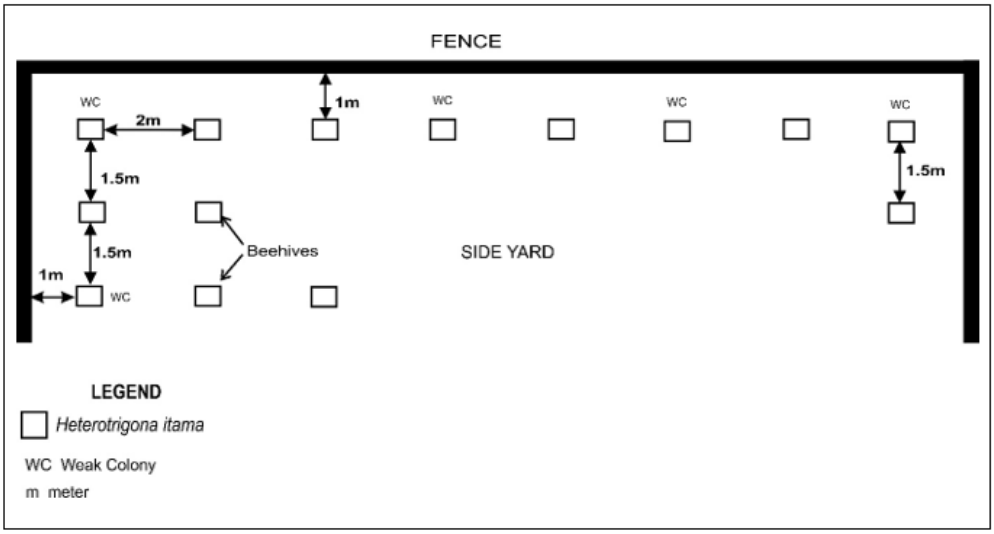

Figure 2. Layout and orientation of hives within the house yard area in Lot 19, Taman Sejati Ujana

Figure 3. A beehive with a roof lined with a rubber mat or aluminium and placed on wood stand at a height of 50 to 60 cm above the ground

follow the marked bees in the studied sites, prior to the beginning of data collection. Twenty individuals of stingless bees from each of the beehives were marked on the thoraces using white correction liquid and then immediately released. This method is described by Peakal and Schiestl (2004). The search for *marked* individuals was done through inspection from tree to tree canopy, tree trunks, agricultural foliage, vegetable farms, gardens, and shrubs, among other areas. This observation was repeated 16 times in each of the sites. To examine the stingless bees' foraging height on the tree canopies, observations were made at close range with a portable 80 x 100 magnification monocular telescope for wildlife watchers (HaleBor model C74153). The flight height by bees was estimated based on the height of the tree canopy visited by foragers, and was measured using ImageMeter software version 3.5.0 (2).

Foraging Behaviour and Time by Marked Bees, and Measurement of Abiotic Factors

The foraging behaviour of bees was observed in front of the hive entrance tube, and recorded 272 times on rain-free days using a Huawei Mate P20 Pro smartphone digital video recorder. A video recording of the foraging traffic from three selected beehives in Lot 19, Taman Sejati Ujana, Sandakan, Sabah was done before the first foraging departure at 5:30 a.m., and ended at 6:30 p.m., after the termination of foraging activities. The foraging activity was determined from the video sequences, using real-time playback, and the counting methods were carried out as described by Kaluza et al. (2016). A stopwatch and a hand tally counter were used to count the number of foragers. To obtain the active foraging data, the number of exiting and returning foragers for the selected three beehives was

measured for three minutes during each hour, from 5:30 a.m. until 6:30 p.m..

To obtain floral or non-floral material data collected by foragers, only the number of returning foragers for each material load was counted for three minutes each hour, from 5:30 a.m. until 6:30 p.m.. These data were then combined according to the following time phases: (i) early morning (5:30 to 9:30 a.m.); (ii) morning to afternoon (10:00 a.m. to 12:30 p.m.); (iii) afternoon to evening (1:00 to 3:30 p.m.); and (iv) evening by to dusk (4:00 to 6:30 p.m.). The foragers returning to the beehives without pollen and resin loads on their tibia were recorded as liquid foragers. These liquid foragers, which were suspected to forage nectar and water, could be distinguished from the rest by having a swollen abdomen (Figure 4). In this study, the nectar and water loads were not individually determined. To test the relationship between bees' foraging patterns and abiotic factors, the temperature (°C) and relative humidity (RH) were measured over two months from May 2019 until June 2019, using a Hobo® U23-002 PV2 data logger that was kept in Site H at Lot 19, Taman Sejati Ujana.

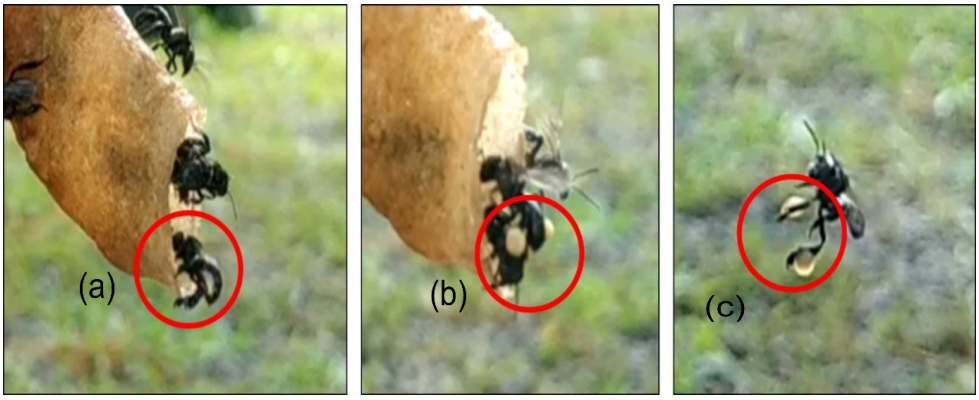

Figure 4. Returning foragers with (a) swollen abdomen, (b) pollen, and (c) resin loads on their tibia

Statistical Analysis

Statistical analysis tests were carried out using Microsoft Excel and IBM Statistics SPSS for Windows, version 24. The data was sorted by sample date and pooled within the study sites to simplify the presentation. All data were tested for normality using Kolmogorov-Smirnov one-sample tests, before applying parametric analyses. Analysis of variance (ANOVA) was used to test whether there was a significant difference in the foraging preferences by marked bees between the study sites; whether there was a significant difference in a variety of content resources collected by foragers; and to test whether there was a significant difference in the type of loads collected by foragers at distinct time phases of the day. To obtain

whether the abiotic factors influence the bees' foraging behaviour in the residential area, the relationships between the active foraging pattern of *H. itama* and phases of daylight cycle, ambient temperature and relative humidity were investigated using Pearson's correlation.

RESULTS AND DISCUSSION

Sites Foraging Preferences of *Heterotrigona itama* within a Residential Area

There was a significant difference in the visitations per day foraged by marked bees among the study sites (Table 2; one-way AVOVA; $F_{7, 319}$ =85.64, $p < 0.001$). Table 2 shows that the marked foragers of *H. itama* most preferred to forage at Site H (average visitations per day = 8.5), followed by Site A (average visitations per day = 8.07), and Site C (average visitations per day = 3). Ciar et al. (2013) reported that *Tetragonula carbonaria* were observed to travel up to 500 m, but preferred to exploit food sources closer to their hive (1 m). Overall, the marked bees of *H. itama* preferred to forage the various resources available within a 500 metres radius from the house yard (Lot 19, Taman Sejati Ujana in Site H), particularly where food sources were more diverse and closer to their hives. Among the known flowering plants visited by *H. itama* in Sites A, C, F, and H were banana, mango, papaya, shrubs, leafy vegetables, citrus, guava, mulberry, chilli, tapioca, coconut, coral vines, *Tunera* sp., *Ixora* sp., *Acacia* sp., *Macaranga* sp., *Melastoma malabathricum*, *Mimosa pudica*, *Ageratum* sp., *Passiflora* sp., *Imperata cylindrica*, and *Mikania micrantha.* The least preferred foraged sites by marked foragers of *H. itama* were Site G (average visitations per day = 0.03), followed by Site D (average visitations per day = 0.38) and Site B (average visitations per day = 0.63). The lower number of marked bees visiting Sites B and D compared to other sites was likely to be influenced by the flowering season of ornamental trees in parks, which rarely occur during the sampling time (Table 2). Whilst for Site G, the marked bees visiting this area was likely because of the dragon fruit flowers blooming in the early morning (6:30 to 7:00 a.m.), which occurred only once throughout the study.

Bees usually prefer to forage in semi-natural habitats consisting of a mixture of natural forest areas, parks and agricultural landscapes, since these habitats provide more diversified food resources (Steffan-Dewenter & Tscharntke, 2001). Table 2 shows that *H. itama* is a generalist species, as they visited all known plant species, regardless of the landscape type around the residential area. Study by Md Zaki and Abd. Razak (2018) in the rubber smallholder environment also found that *H. itama* was able to forage more than 29 species of identified plants from 22 families. Landaverde-González et al. (2017) also found that the stingless bee species of *Partamona bilineata* in Guatemala could survive well and efficiently forage floral resources in modified landscape areas. During the study, *H. itama* was also observed to be able to find pollen in the

canopy of pioneer trees of *Macaranga* sp. on Site C, and coconut trees on Site A, which have a tree height of about 10 m. Therefore, this suggests that the height of the tree was not a barrier for this species to gather material resources from the canopy level. This situation shows that this species is not only able to exploit various food sources in urban areas, but it was also able to fly high in search of food in the tree canopy.

Table 2

Sites foraging preferences by marked foragers of Heterotrigona itama *from Lot 19, Taman Sejati Ujana (Site H), measured for 40 days*

Study sites	Straight line distance measurement from Site H (m)	Total marked foragers (n)	Average visitations per day by marked foragers (SE)
A	50	323	8.07^{d} (0.53)
B	60	25	0.63^{ab} (0.17)
C	95	120	3^{c} (0.43)
D	90	15	0.38^{a} (0.99)
E	350	55	1.37^{ab} (0.26)
F	500	75	1.88^{bc} (1.67)
G	1000	1	0.03^{a} (0.03)
H	0 – 40	340	8.5 (0.66)

Note. Letters indicate significance differences measured by post hoc Tukey B test ($p < 0.05$). SE = Standard error, A = Private agriculture area 1, B = Park 1, C = Unmanaged landscape, D = Park 2, E = Natural forest, F = Private agriculture area 2, G = Private garden, and H = Taman Sejati Ujana

Foraging Behaviour of *Heterotrigona itama* in A Residential Area

Type of Materials Resources Foraged by the Marked Bees. There was a significant difference between the variety of content resources collected by bees (Figure 5; one-way ANOVA; $F_{2,59} = 8.95$, $p < 0.001$). Figure 5 shows that the nectar or water sources was the highest material (51.39%), which was brought back to the hive by foragers, followed by resin (34.73%) and pollen (13.87%). The stingless bee workers usually foraged pollen, resinous plants, and nectar or water sources, to meet the needs of the colony (Gaona et al., 2019). Nectar, which is converted by bees into honey, serves as the primary source of carbohydrates to supply energy for the colony's survival and foraging activities (Kajobe, 2007). The higher number of nectar or water foragers found in this study shows that *H. itama* was likely more attracted to both nectar-rich inflorescence and water resources. Studies on the mineral-foraging

of *Trigona silvestriana* in Costa Rica showed that foragers were highly attracted to essential minerals from mineralised water than deionised water (Dorian & Bonoan, 2016). These mineral salts such as sodium, magnesium and potassium which obtained from water, are crucial for developing larvae and brood food (Lau & Nieh, 2016). Prominent studies about water collection by stingless bees are those by Bijlsma et al. (2006), Cham et al. (2019), and Roubik (2006). In this study, *H. itama* was also observed to have taken water from the wet soil around the house yard. Roubik (1989) indicated that other non-floral materials collected by bees comprise oils, honeydew, sap, gums, wax, plant parts, mud, fungi, spores, and water. Lorenzon and Matrangolo (2005) reported that 12 species of stingless bees in the Caatinga region were observed to forage non-floral resources such as muddy water to seek mineral salts. According to Requier and Leonhardt (2020), the bees need non-floral resources for nest building, defence, protection, and colony health. Dreisig (2012) found that bees continued to visit the flowers that have massive amounts of nectar, but left an unrewarding plant quickly.

Figure 4 shows that the foragers returning with fluid loads, which could be nectar or water stored in their crops, did not bring pollen or resin together. The liquid loads of resin and pollen were deposited on the corbiculae structure that is located in the hindlegs tibia of foragers (Figure 4). For stingless bees, plant resins are crucial resources for nest defence and construction (Leonhardt & Bluthgen, 2009; Wallace & Lee, 2010). Additionally, the resin has rich antifungal, antibacterial, and antiviral properties (Leonhardt &

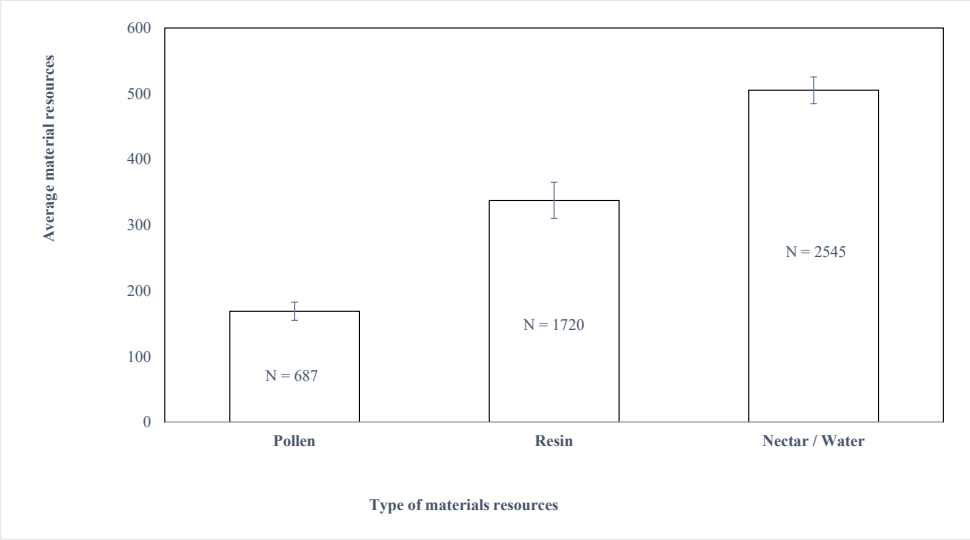

Figure 5. Type of materials resources collected by *Heterotrigona itama* in the environment

Note. Letters indicate significance differences measured by post hoc Tukey B test ($p < 0.05$)

Bluthgen, 2009). This species was found to have taken resin from tapioca leaf stalks and resinous liquids on the bark of mango trees planted at about 50 m from the house yard. Simone-Finstrom et al. (2017) found that the increase in resin foraging activities by *Apis mellifera* was related to nest development, and acted as a defence mechanism against parasites and predators attacking the colony. According to Leonhardt and Bluthgen (2009), foragers preferred floral resin that does not harden quickly, so that bees can reuse it for construction within the nest. The pollen from flowering plants is vital as a source of protein and lipids essential for growth, development, and reproduction of the bee colony (Karuppasamy & Jeyaraaj, 2016). Unlike nectar and resin, pollen is a limited resource that cannot be continuously replenished by plants, and can be quickly depleted during the day (Di Pasquale et al., 2016). Thus, this likely explains why pollen had the lowest average collected by foragers in this study.

Foraging Behaviour in Relation to Abiotic Factors and Time Phases. *Heterotrigona itama* started foraging at 5:30 a.m., and the foraging activity fluctuated during the day from the morning until the evening at 6:30 p.m. (Figure 6). This study indicates that the active foraging pattern of *H. itama* was negatively correlated to the time phases of the day (Pearson's correlation; r_p = -0.371, $p < 0.001$). This phenomenon was contributed by the three peaks of foraging hours, which reached a peak in the early morning (6:30 to 8:00 a.m.), moderately peaked towards the evening (2:30 to 3:30 p.m.), and was the highest towards the afternoon (10:30 a.m. to 12:00 p.m.). A study on similar species by Basari et al. (2018) in Terengganu, Peninsular Malaysia showed that the peaked foraging hours occurred at 7:00 a.m. to 12:00 p.m., and from 2:00 p.m. to 3:00 p.m.. Both studies of *H. itama* conducted in the east and west of Malaysia showed a peak of foraging activities in the morning. Morning peaked foraging for both floral and non-floral resources have also been reported in many other species of tropical stingless bees, including *Tetragonula collina*, *Tetragonula rufibasalis*, *Tetragonula melanocephala*, and *Tetragonula melina* (Nagamitsu & Inoue, 2002). Some stingless bees (i.e. *Trigona sapiens* and *Trigona hockingsi*) may show early morning and mid-afternoon peaks in floral or non-floral materials collection (Wallace & Lee, 2010). Differences in hours of foraging activity within a day may vary depending on resource availability, distance, and ecological factors (Basari et al., 2018; Wallace & Lee, 2010). The foraging activity can also correspond to both plant pollination-related characteristics and eusocial bees (Lichtenberg et al., 2016). According to Lau et al. (2019), the interaction between plants and insect pollinators could occur at a certain period during the day that can affect various ecological processes in the environment.

The ambient temperature and relative humidity were not the main

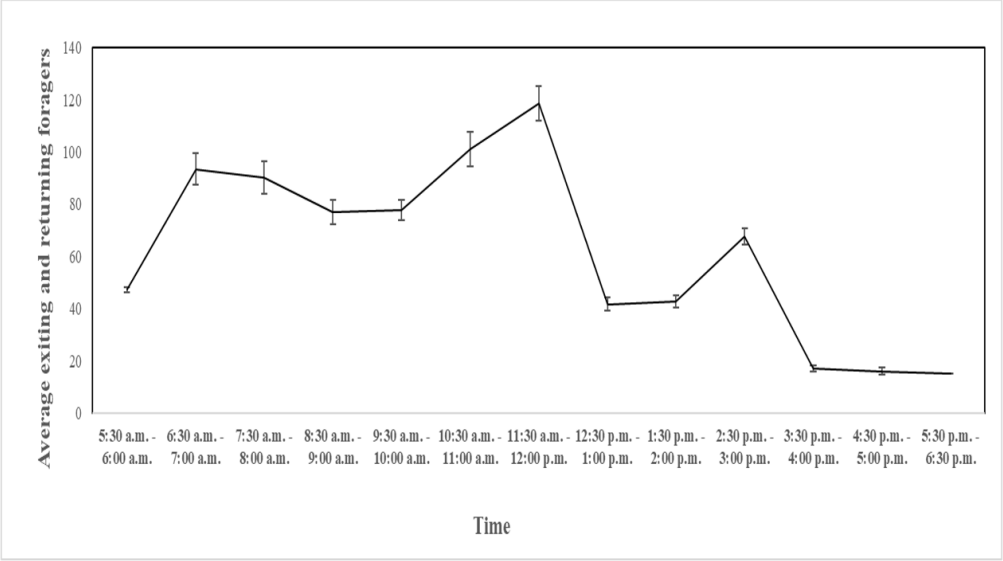

Figure 6. *Heterotrigona itama* foraging pattern during a day

factors influencing the overall average of foragers exiting and returning to the hives [temperature (Pearson's correlation: r_p = -0.06, N = 13, p = 0.84) and humidity (Pearson's correlation: r_p = 0.03, N = 13, p = 0.92)]. Similar foraging patterns were also observed in *Melipona asilvai*, whereby temperature and humidity were not the main factors influencing the bees' foraging activity (do Nascimento & Nascimento, 2012). However, a closer examination of the data in Table 3 show that the activity of *H. itama* started to peak from 10:30 a.m. until 12:00 p.m., when the relative humidity was between 62.8% to 67.8%, and the temperature was between 31.7°C to 32.6°C (Table 3). The ideal temperature and relative humidity ranges for foraging activity vary between bees of different species, some of which may be affected by changes in microclimatic conditions (Abou-Shaara et al., 2012; Hilário et al., 2001; Li et al., 2019). Li et al. (2019) found that *Apis mellifera* and *Apis cerana* were intolerant to high temperature ranges of 54 to 60°C, and 57 to 60°C, respectively, particularly when their body water loss rate increases. Abou-Shaara et al. (2012) found that Yemeni honeybees, *Apis mellifera jemenitica*, were more tolerant to high temperature and low humidity conditions in the desert, compared to Carniolan honeybees, *Apis mellifera carnica*. In this study, *H. itama* seem to be better adapted to the heat and humidity conditions of urban environments, making them suitable for rearing in the house yard (Table 2). A closer examination of the data in Table 3 shows that the optimum temperature and humidity conditions for active tropical stingless bee activities in the urban environment range from 25.1 to 33.3°C, and 61.8 to

Table 3

Foraging behaviour of Heterotrigona itama *in relation to temperature and relative humidity, measured from May 2019 until June 2019 in Lot 19, Taman Sejati Ujana*

Observed time	Average of exiting and returning foragers (SE)		Average temperature (°C) (SE)		Average relative humidity (%) (SE)	
5:30 a.m. – 6:00 a.m.	47.3	(2.1)	25.2	(0.10)	94.3	(0.48)
6:30 a.m. – 7:00 a.m.	93.5	(12.2)	25.1	(0.03)	95.0	(0.41)
7:30 a.m. – 8:00 a.m.	90.3	(12.4)	26.7	(0.24)	87.3	(0.75)
8:30 a.m. – 9:00 a.m.	77.2	(9.4)	28.9	(0.03)	78.8	(1.25)
9:30 a.m. – 10:00 a.m.	77.7	(7.6)	31.0	(0.09)	71.8	(0.25)
10:30 a.m. – 11:00 a.m.	101.1	(13.4)	31.7	(0.10)	67.8	(0.85)
11:30 a.m. – 12:00 p.m.	118.5	(13.2)	32.6	(0.13)	62.8	(0.25)
12:30 p.m. – 1:00 p.m.	41.8	(5.1)	33.2	(0.06)	61.8	(0.25)
1:30 p.m. – 2:00 p.m.	42.8	(5.0)	33.3	(0.09)	61.8	(0.25)
2:30 p.m. - 3:00 p.m.	67.5	(6.1)	33.1	(0.03)	71.8	(0.25)
3:30 p.m. – 4:00 p.m.	17.1	(2.3)	31.8	(0.06)	69.0	(0.41)
4:30 p.m. – 5:00 p.m.	16.1	(2.4)	30.4	(0.27)	74.8	(0.25)
5:30 p.m. – 6:30 p.m.	15.0	(0.0)	27.9	(0.03)	82.8	(0.25)

Note. SE = Standard error

95%, respectively. Li et al. (2019) stated that the survival rates of *A. mellifera* and *A. cerana* at high temperatures in the environment depend on the duration of exposure and relative humidity. Heat stress in the environment significantly reduces the colony growth of bees and their workers' survival (Abou-Shaara et al., 2012; Hilário et al., 2000).

There was a significant difference in foraging time phases by returning foragers for collecting resin (one-way ANOVA; $F_{3,19} = 4.50$, $p = 0.02$) and nectar or water (one-way ANOVA; $F_{3,19} = 4.31$, $p = 0.02$) (Figure 7). However, there was no significant difference in foraging time phases found for pollen (one-way ANOVA; $F_{3,19} = 1.41$, $p = 0.28$) (Figure 7). Thus, stingless bees' foraging behaviour could be influenced by the availability and quality of floral or non-floral materials in the environment (Roubik et al., 1995), which can vary depending on the time of day. Ghosh et al. (2020) indicate that the trend of active bees' foraging activity depends on the time during which foragers can obtain the highest reward from their visit to a flower. Figure 7 shows that peaks of *H. itama* foraging for resin and nectar or water occur from 10:00 a.m. to 12:30 p.m. (morning to noon), slightly decreased from 1:00 to 3:30 p.m. (noon to evening), and were lowest towards 4:00 p.m. to 6:30

p.m. (evening to dusk). The peaks of pollen collection occurred early in the morning, while a fluctuation in the peaks occurred in the afternoon and evening. Some authors demonstrated the same pattern for stingless bees, whereby the activity of foraging peaks in the morning, when a greater availability of food rewards are available (Roubik et al., 1995; Roubik & Aluja, 1983; Roubik & Buchmann, 1984). Fidalgo and Kleinert (2007) found that stingless bees, *Melipona rufiventris*, can optimise their foraging behaviour by learning the times of the day during which flowers secrete pollen. Some species of stingless bees can shift the time of pollen collection during the day, which could be correlated to the floral phenology of the area such as a blooming peak (Bruijn & Sommeijer, 1997).

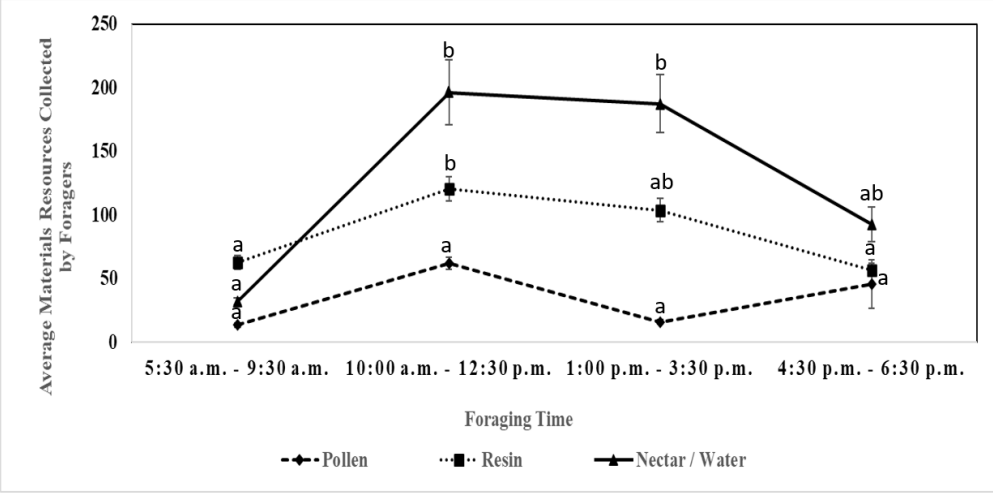

Figure 7. Type of materials resources collected by returning foragers in relation to phases of time during a day

Note. Letters indicate significance differences measured by post hoc Tukey B test ($p < 0.05$)

CONCLUSION

This study investigated the foraging ecology of *Heterotrigona itama* in the house yard, which has not being well-explored in prior work. The colonies of *H. itama* were kept in a house yard, and were shown to be a generalist species because they were able to exploit a wide variety of food resources and landscape types around the residential area. However, they preferred to forage various resources available within a 500-metre radius from the house yard, particularly where food sources were more diverse and closer to their hives. The high number of nectar or water foragers found in this study shows that *H. itama* were more attracted to both nectar-rich inflorescence and water resources, compared to resin and pollen sources. The foraging activity by *H. itama* at the house yard was generally active

throughout the study period, and the ambient temperature and humidity were not the main factors influencing the bees' foraging activity. The *H. itama* foraging time phases were influenced by the availability and quality of food resources in the environment, which varied depending on the time of day. Thus, this study highlighted some essential criteria that needed to be considered for beekeeping in residential areas, which include an in-depth understanding of the species' foraging ability in exploiting food resources in an urban environment, and the viability of the species to adapt to urban microclimatic conditions.

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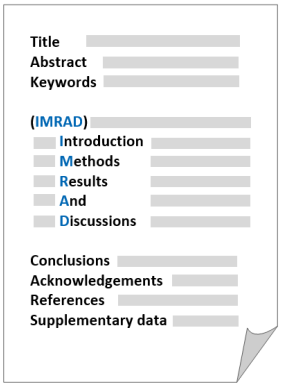

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Journal article with 1-2 authors	**Information prominent' (the author's name is within parentheses):** … (Carolan, 2020) … Or **'Author prominent' (the author's name is outside the parentheses):** Carolan (2020) …	Carolan, M. (2020). Automated agrifood futures: Robotics, labor and the distributive politics of digital agriculture. *The Journal of Peasant Studies*, *47*(1), 184-207. https://doi.org/10.1080/03066150.2019.1584189
Journal article with 3 or more authors	*For all in-text references, list only the first author's family name and followed by* 'et al.' **Information prominent' (the author's name is within parentheses):** … (Kumar et al., 2020) … … (Kumari et al., 2019) … Or **'Author prominent' (the author's name is outside the parentheses):** Kumar et al. (2020) … Kumari et al. (2019) …	Kumar, A., Padhee, A. K., & Kumar, S. (2020). How Indian agriculture should change after COVID-19. *Food Security*, *12*(4), 837-840. https://doi.org/10.1007/s12571-020-01063-6 Kumari, R., Choudhury, D., Goswami, S., & Dey, N. (2019). Physiological, biochemical, and molecular screening of selected upland rice (Oryza sativa L.) lines from eastern India. Bulletin of the National Research Centre, 43(1), 56. https://doi.org/10.1186/s42269-019-0087-9
Journal article with more than 20	**Information prominent' (the author's name is within parentheses):** … (Tobler et al., 2017) … Or **'Author prominent' (the author's name is outside the parentheses):** Tobler et al. (2017) …	Tobler, R., Rohrlach, A., Soubrier, J., Bover, P., Llamas, B., Tuke, J., Bean, N., Abdullah-Highfold, A., Agius, S., O'Donoghue, A., O'Loughlin, I., Sutton, P., Zilio, F., Walshe, K., Williams, A. N., Turney, C. S. M., Williams, M., Richards, S. M., Mitchell, N. ... Cooper, A. (2017). Aboriginal mitogenomes reveal 50,000 years of regionalism in Australia. Nature, 544(7649), 180-184. https://doi.org/10.1038/nature21416
Journal article with an article number	**Information prominent' (the author's name is within parentheses):** … (Bougnom et al., 2020) … Or **'Author prominent' (the author's name is outside the parentheses):** Bougnom et al. (2020) …	Bougnom, B. P., Thiele-Bruhn, S., Ricci, V., Zongo, C., & Piddock, L. J. V. (2020). Raw wastewater irrigation for urban agriculture in three African cities increases the abundance of transferable antibiotic resistance genes in soil, including those encoding extended spectrum β-lactamases (ESBLs). *Science of The Total Environment*, *698*, 134201. https://doi.org/10.1016/j.scitotenv.2019.134201
Journal article with missing information	**Information prominent' (the author's name is within parentheses):** … (Pryce et al., 2018) … … (Saberi et al., 2018) … … (Rahman et al., 2020) …	**Missing volume number** Pryce, J., Choi, L., Richardson, M., & Malone, D. (2018). Insecticide space spraying for preventing malaria transmission. *Cochrane Database of Systematic Reviews*, (11), CD012689. https://doi.org/10.1002/14651858.CD012689.pub2

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Several works by the same author in the same year	**Information prominent' (the author's name is within parentheses):** … (Lim et al., 2019a, 2019b) … Or **'Author prominent'(the author's name is outside the parentheses):** Lim et al. (2019a, 2019b) …	Lim, L. W. K., Chung, H. H., Chong, Y. L., & Lee, N. K. (2019a). Enhancers in proboscis monkey: A primer. *Pertanika Journal of Tropical Agricultural Science*, *42*(1), 261-276. Lim, L. W. K., Chung, H. H., Chong, Y. L., & Lee, N. K. (2019b). Isolation and characterization of putative liver-specific enhancers in proboscis monkey (*Nasalis larvatus*). *Pertanika Journal of Tropical Agricultural Science*, *42*(2), 627- 647.
	Newspaper	
Newspaper article – with an author	… (Morales, 2020) ... Or ... Morales (2020) ...	Morales, C. (2020, November 13). Scientists destroyed a nest of murder hornets. Here's what they learned. *The New York Times*. https://www.nytimes.com/2020/11/13/us/murder-hornets-us.html
Newspaper article – without an author	("Japan bird flu outbreak", 2020). OR "Japan bird flu outbreak" (2020) … Use a shortened title (or full title if it is short) in Headline Case enclosed in double quotation marks.	Japan bird flu outbreak spreads to farm in fourth prefecture. (2020, December 01). *The Straits Times*. https://www.straitstimes.com/asia/east-asia/japan-bird-flu-outbreak-spreads-to-farm-in-fourth-prefecture
	Dissertation/Thesis	
Published Dissertation or Thesis References	… (Sutradhar, 2015) ... Or … Sutradhar (2015) ...	Sutradhar, M. (2015). *Metagenomic analysis of rhizospheric microbial diversity in rice grown under irrigated and aerobic condition* [Master's thesis, University of Agricultural Sciences]. KrishiKosh. http://krishikosh.egranth.ac.in/handle/1/5810027635
Unpublished Dissertation or Thesis References	… (Rahman, 2017) ... Or … Rahman (2017) ...	Rahman, F. (2017). *Ecological assessment of the reintroduced milky stork population in Malaysia* [Unpublished Doctoral dissertation]. Universiti Putra Malaysia.
	Conference/Seminar Papers	
Conference proceedings published in a journal	… (Dotaniya & Meena, 2015) … Or Dotaniya and Meena (2015) …	Dotaniya, M. L., & Meena, V. (2015). Rhizosphere effect on nutrient availability in soil and its uptake by plants: A review. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *85*(1), 1-12. https://doi.org/10.1007/s40011-013-0297-0
Conference proceedings published as a book chapter	… (Kurbatova et al., 2019) … Or Kurbatova et al. (2019) …	Kurbatova, S. M., Aisner, L. Y., & Naumkina, V. V. (2019). Some aspects of the essence and legal regulation of agriculture digitalization as one of the priorities of modern state policy of agriculture development. In *IOP conference series: Earth and environmental science* (Vol. 315, No. 3, p. 032021). IOP Publishing. https://doi:10.1088/1755-1315/315/3/032021

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Online	… (Melanie et al., 2017) … Or Melanie et al. (2017) …	Melanie., Rustama, M. M., Kasmara, H., Sejati, S. A., Fitriani, N., & Madihah. (2017, October 25-26). *Pathogenicity of* Helicoverpa armigera *polyhedrosis sub culture virus (*Ha*NPV$_1$) on* Spodoptera litura *Fabricius* [Paper presentation]. Prosiding Seminar Nasional Penelitian dan Pengabdian pada Masyarakat (SnaPP) 2017 Sains dan Teknologi, Bandung, Indonesia. http://proceeding.unisba.ac.id/index.php/sains_teknologi/article/view/988/pdf
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