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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.

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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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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.

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To publish journals of international repute.

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Citing journal articles

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

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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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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).

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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:

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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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Contents

Foreword

Welcome to the third 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 11 articles; two review articles and the rest are regular articles. The authors of these articles come from different countries namely Indonesia, Malaysia, and Thailand.

A selected article entitled "Small Pteropodid Bats are Important Pollinators of Durian in Terengganu, Malaysia" reported the role of small pteropodid bats as pollinating agents to flowering durian trees. Samplings were conducted in April 2018 to record three small pteropodid bats, namely, *Cynopterus brachyotis*, *Cynopterus horsfieldii*, and *Eonycteris spelaea*, visiting the flowers of two durian species, *Durio zibethinus* and *Durio lowianus* at agricultural areas in Hulu Terengganu. Captured bats were swabbed for conspecific pollen load on their bodies to determine their potential role as pollinators. Based on the observation, *E. spelaea* was likely to be a more important pollinating agent since this species was frequently captured near the flowering trees and found to carry a significantly high number of conspecific pollen grains on their bodies. The detailed information of this article is available on page 583.

A review paper entitled "The Potential of Silicon in Improving Rice Yield, Grain Quality, and Minimising Chalkiness" discussed the ability to uptake silicon (Si) and its benefits on rice. An update on the potentials of Si in improving the rice yield and grain quality, including Si's ability to minimise grain chalkiness, was presented as well. The further details of this study are found on page 655.

Anto Budiharjo and his teammates from Diponegoro University evaluated the improvement in the quality and concentration of RNA after the optimised TRIzol-based treatment. One-month-old tomato (*Solanum lycopersicum*) stem was used in this research. Several optimisation steps, such as the increment of the initial sample amount, twice chloroform extraction, overnight precipitation at low temperature, and three times final washing with ethanol, were practised. A higher quantity and quality of extracted RNA was obtained. Full information of this study is presented on page 673.

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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Habitat Use and Movement Activity of *Neolissochilus soroides* and *Channa lucius* during Post Inundation of Tembat Reservoir, Hulu Terengganu

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ABSTRACT

The drastic changes in hydroelectric reservoir development created a completely new ecosystem that affects the river, particularly in the inundated area. In this study, five *Neolissochilus soroides* and *Channa lucius* were surgically implanted with a unique coded acoustic transmitter to observe the habitat utilisation and movement activity in Tembat Reservoir after the inundation process. All of the individuals were released into the transition zone of the reservoir and observed using passive and active acoustic tracking devices from April to December 2018. Kruskal-Walis test showed no significant difference between the average size of core area for *N. soroides* and *C. lucius*, $x^2(1) = 1.320$, $p = 0.251$. The home range also showed a similar result for *N. soroides* and *C. lucius* where there was an insignificant difference, $x^2(1) = 0.273$, $p = 0.602$. However, duration wise, *N. soroides* spend more time in the transition zone, R1 (M = 2.71 hrs, SE = 0.38), and *C. lucius* in the riverine zone, R5

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(M = 7.14 hrs, SE = 6.13) and R6 (M = 3.85 hours, SE = 6.36). From the active tracking survey, PCA identified three (62.32%) and four (71.19%) components with eigenvalues greater than 1 for *N. soroides* and *C. lucius*, respectively. Three most important habitat parameters for *N. soroides* were the size of river (r = 0.97), existence of canopy cover (r = 0.77), and substrate type (r = 0.79).While, for *C. lucius* were mesohabitat type (r = 0.79), distance to riffle (r = 0.97), existence of canopy cover (r = 0.90), and elevation (r = 0.90). A 24-hour movement frequency analysis for both species revealed two active hours of movement at dusk and dawn for both species. From the study, it is known that *N. soroides* and *C. lucius* respond differently towards the inundation of the reservoir. The findings in this study can be implemented for effective aquatic management and conservation plan to ensure sustainable dam development.

Keywords: Fish movement, fish telemetry, habitat use, hydroelectric impacts, post-inundation

INTRODUCTION

Inundation of water catchment areas in the tropical rainforest for hydroelectric stations is known to have a devastating effect on the environment. Many studies have described the ecological implications, and the effect is more pronounced in tropical water catchment areas (Fearnside, 2014; Huang & Li, 2016). These areas are often subject to hydroelectric power development due to geographical landscape and topographic suitability (Zafirah et al., 2017). The construction and pre-impoundment processes, including forest clearing, significantly alter the initial aquatic environment. The most prominent modification is the multidimensional change of the natural water body in the catchment (Zeiringer et al., 2018). The aquatic landscape is usually transformed into an open system with less or no canopy, depending on the volume of the water. It can be divided into three longitudinal systems: riverine (lotic), transition, and lacustrine (lentic) zones (Schmutz & Moog, 2018). The modification can also involve stratification of the water column in deeper areas where anoxic conditions can occur towards the bottom substrate, making the environment less habitable for aquatic fauna (Kimmel & Groeger, 1984).

The effects of impoundment on the native river species have gained substantial attention (Kano et al., 2016). In this newly developed lentic habitat, the fish assemblage can be modified qualitatively and quantitively through extermination of native fish species, reduction of habitat diversity, increasing densities of indigenous species, and introducing non-native species (Zhong & Power, 1996). In addition, the impoundment of downstream areas can influence the fish assemblage through changes in temperature, flow regime, sediment loads, and turbidity of the area (Li et al., 2016). Although in-depth studies of impacts on the ecology of aquatic fauna have been widely discussed, there is still a need to understand more about the utilisation of habitat and movement of fish fauna in a newly inundated riverine system, as this

subject is poorly documented. Identifying habitat use characteristics and movement patterns is essential for restoring fish fauna and management planning (Clarke et al., 2007).

Neolissochilus soroides is a cyprinid found in Southeast Asia, particularly the Malay Archipelago and Borneo. It is a mahseer species indicative of high altitude, fast-flowing, and excellent water quality (Khaironizam et al., 2015). *Channa lucius*, or the forest snakehead, is a common species in the Tembat River and found throughout southeast Asia. Unlike other channid species common in peat swamps and stagnant water, *C. lucius* can be found in fast-flowing clear water at higher altitudes (Ambak et al., 2012). These species are native to Tembat River and a good candidate to represent the black and white water species that may respond to the effect of the inundation differently.

Despite habitat degradation and exploitation being threats to both species, the species are listed as the least concern (LC). Nevertheless, there are currently no published data on the behaviour and movement patterns of these species. Most of the studies conducted on *N. soroides* and *C. lucius* are of their biological and aquaculture potential (Azrita et al., 2015; Khai et al., 2015). The distinctive differences in life-history requirements of both species are very important to recognise, especially in identifying areas for biodiversity conservation (Asaad et al., 2017). A mahseer species, such as *N. soroides*, are an umbrella species, which means, protecting its habitat will benefit many other species under its trophic level (Pinder et al., 2019).

While *C. Lucius,* is widely tolerant to water quality and can be found in a very low pH environment, this species prefers pristine forested rivers. Moreover, its sedentary characteristics are a great indicator of microenvironmental change (Ambak et al., 2012). This study provides a preliminary assessment of movement pattern and habitat utilisation for both species within Tembat Reservoir. The knowledge acquired from the study of habitat utilisation and movement activity can inform us how the dam developments impact the ichthyofauna of a local river. Nevertheless, this research is intended to be applied in the management of conservation and habitat rehabilitation efforts. Specifically, these preliminary data can aid in establishing protection zones, such as fish sanctuaries or no-take zones.

This study aims to evaluate habitat utilisation and movement activity of *N. soroides* and *C. lucius* in the transition zone of a small hydroelectric reservoir in the Tembat River system. The specific objectives are: (i) to identify spatial habitat use through the determination of home range and core range area, (ii) to determine environmental variables associated with species detection, (iii) to observe the time spent at different locations within the river, and (iv) to observe the 24-hour movement activity pattern.

MATERIALS AND METHODS

Study Area

The study was conducted in the Tembat

River, Hulu Terengganu, Malaysia, with a pre-experimental study beginning in January 2018 and acoustic tracking conducted from April to December 2018. The Tembat River is part of the Hulu Terengganu Hydroelectric Project (HTHEP), involving approximately 90.834 km^2 with two catchment areas: Puah and Tembat (Figure 1). The climate in this area is equatorial monsoon-type, with a high temperature and year-round rainfall. Almost half of the annual rainfall is recorded during November, December, and January, coinciding with the northeast monsoon (Tenaga Nasional Berhad Research [TNBR], 2007). The study was conducted in the transition zone of the Tembat Reservoir, part of the riverine system.

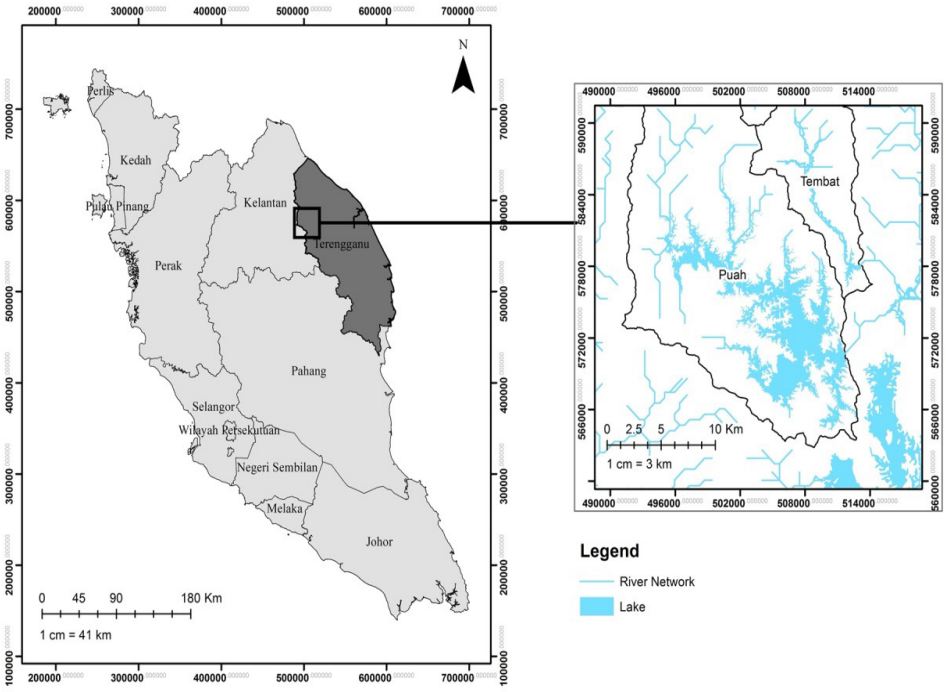

Figure 1. The Tembat Reservoir is located in Tembat catchment Hulu Terengganu, Malaysia which the reservoir is adjacent to the Puah catchment. Both of the catchment is part of Hulu Terengganu Hydroelectric Project (HTHEP)

Fish Capture and Acoustic Transmitter Insertion

Five individuals of *N. soroides* and *C. lucius* were obtained from the Tembat River. *Neolissochilus soroides* were captured using traps baited with fermented palm oil kernels in the Tembat River. At the same time, *C. lucius* were caught by handlines baited with live

juvenile frogs or were fished using artificial lures and casting techniques. All individuals selected weighed more than 550 g, allowing the 3.7 g acoustic tag to be safely implanted. The transmitters were 26 mm long and 9 mm in diameter. Each of the VEMCO V9 transmitters is uniquely codded so that the passive and active receivers can identify the individuals. Based on Weber et al. (2009), the surgical insertion of the transmitters into the peritoneal cavity was carried out using a modified method. Before surgery, individuals were sedated in a clove oil bath. The surgeries lasted for 10-15 minutes and were performed in a container with continuous irrigation of anaesthetics on the gill. Non-absorbable sutures were used to close the incision, and antibiotics were applied to prevent infections. After the surgery, individuals were kept in a 3 m x 1 m fibreglass tank with aggressive aeration for immediate recovery progress. Fish were released within the transition zone of the Tembat River on four occasions: 18 April 2018, 19 April 2018, 31 May 2018, and 4 June 2018. The individuals were released back into the river within 14-30 days after the surgery.

Movement and Habitat Utilisation

Two methods of tracking were used in this study: passive and active tracking. The passive tracking was conducted using six VR2W receivers units deployed within the reservoir. Each receiver station is known as R. VR2W units were placed at 300 m to 800 m intervals between each unit in the river. As determined in a preliminary study, the 100% detection range of VR2W receivers was 66.8 m from the receiver. The deployment areas include a riverine (R5 and R6), transition (R2, R3, R4), and lacustrine zones (R1, R2). The receivers at each location recorded the arrival and departure times of all the uniquely coded IDs of the tagged fish within range, assuming that the signal was unobstructed. The VR2W receivers received pings every 30 seconds from the transmitters in the individuals. The VR2W receivers were deployed underwater for three months (April, May, and June). Data were downloaded using VUE® software (Halifax, Canada).

Active tracking was conducted at least twice each month using VEMCO V100 active tracker and an omnihydrophone. In addition, a single active tracking event was conducted for at least three days from April to December. Active tracking was conducted only during daylight hours as night tracking presents a research limitation as it was unsafe due to active wildlife movement in the Tembat catchment. Tagged individuals were assumed to be within a 5 m radius of the location detected when the signal strength was at its maximum (i.e. 70-90 dB) (Fetterplace et al., 2016). Ten environmental parameters, as listed in Table 1, were observed during individual detection, and the location coordinate was recorded using a Garmin 64s GPS device (Kansas, United States of America).

Table 1

The habitat parameters observed during active tracking in Tembat Reservoir

No.	Parameters and details	Unit	Data type	Definition and method of sampling
1	Type of mesohabitat 1 = Riffle 2 = Pool 3 = Run 4 = Lake	4 types	Categorical	The definition of mesohabitat type are observedbased on hydrology, and physical morphology of the river as recommended by (Baltz, 1990)
2	Type of substrate 1 = Boulder 2 = Cobbles/Pebbles 3 = Sand 4 = Silt	4 types	Categorical	Determined based on the Wentworth particle-size scale (Baltz, 1990). Substrate at deeper area were observed using a underwater camera with monitor on site
3	Existence of tree canopy 0 = Absent 1 = Present	2 types	Categorical	Determined based on the existence of vegetation at bank that provide shade or refuge. Vegetation coverings include overhanging vegetation, root wads or undercut banks (Rutherford et al., 1997)
4	Existence of submerge large wood 0 = Absent 1 = Present	2 types	Categorical	Submerge Large Woods are defined as remnants of log from logging activity that maybe fully or partially submerge in the river (Dolloff & Warren, 2003). Any submerge large wood in the 5 m radius of area where fish were detected

Table 1 *(Continued)*

No.	Parameters and details	Unit	Data type	Definition and method of sampling
5	Type of bank 1= Boulders 2=Cobbles/Pebbles 3 = Sand 4 = Silt	4 types	Categorical	The type of bank observed were parallel to the location fish were detected and based on the particle-size scale mentioned by (Baltz, 1990)
6	Width of river	meters (m)	Nominal	Width of the river were measured using a laser range finder by calculating the distance from fish location to both banks
7	Distance to riverbank	meters (m)	Nominal	Distance to riverbank were calculated by measuring the shortest distance of fish location to the riverbank
8	Distance to riffle	meters (m)	Nominal	Distance to riffle were determined using ArcGIS where all of the riffle location in the river were mapped and the nearest riffle location from the fish location were determined
9	Distance to lake	meters (m)	Nominal	Location where the lacustrine zone or the lake started were plotted in ArcGIS and distance were measured from the location fish were detected to the point
10	Depth	meters (m)	Nominal	Depth of the area where fish were located were determinen using a sonar depth finder

Data Analysis

Home range size estimations were analysed using the Fishtracker 10.1 toolbox extension in ArcGIS 10.5 software. Fishtracker uses a least-cost path to determine the travel speed between sequential observations and convert it to per-segment travel times (Laffan & Taylor, 2013). The movement rate was then used in a standard kernel density analysis. In this study, the sequential arrival and departure detection data from each receiver, complemented with active tracking data, provided the substance of the home range estimate. The product of this analysis is a surface area estimate for a home range and a core range.

Individual time spent at each receiver station (R) was observed by analysing the arrival and departure time logged. In addition, the average 24-hour movement activity of *N. soroides* and *C. lucius* was determined using bi-hourly movement count data from VR2W receivers. Active tracking was conducted from April to December simultaneously with passive tracking. During active tracking, ten environmental parameters were observed at every individual location detection. Principal component analysis (PCA) was used to reduce the dimensionality of the environmental variables recorded in this study. Matrices of ten parameters at every location where the individuals were detected using active tracking were constructed for both species to run the PCA. PCA was conducted in Statistical Package for the Social Sciences (SPSS) version 26.0, using varimax rotation with 25 maximum iterations. The Kaiser-Guttman criterion (Guttman, 1954) and the broken-stick criterion (Frontier, 1976) were applied as stopping rules for determining the number of principal components extracted. These rules are based on average test statistic values where eigenvalues that are larger than the average value expected under the null hypothesis are used to assess the relative interpretability of the ordination results. In this study, principal components (PCs), which have eigenvalues greater than one, were considered to represent significant correlations of variance and were extracted (Jackson, 1993). In the rotated component matrix, the highest environmental variables correlation of greater of the component were highly influential.

RESULTS

Home and Core Range of *Neolissochilus soroides* and *Channa lucius*

All of the individuals in this study were successfully tracked within the river between April and December 2018. The detection span for each individual ranged from 10 days to 244 days. Individual ID 16285 had the shortest detection span, which only lasted for ten days. Therefore, this individual was assumed to be out of the detection range for the rest of the tracking period. The detection span for the rest of the individuals ranged from 193 to 219 days (*C. lucius*) and 132 days to 244 days (*N. soroides*).

The 50% kernel estimation density (KED) core area size of *N. soroides* ranged from 15283 m^2 to 86563 m^2. (Figure 2),

while *C. lucius* ranged from 36545 m^2 to 89578m^2 (Figure 3). Kruskal-Wallis test conducted to compare the core area size between the species was insignificant, $x^2(1) = 1.320$, $p = 0.251$. While the 90% KED home range area ranged from 28078 m^2 to 155848 m^2 for *N. soroides*, and *C. lucius* ranged from 68399 m^2 to 161425 m^2, which also tested insignificant, $x^2(1) = 0.273$, $p = 0.602$ (Table 2). These results suggest that the home range and core range size of *C. lucius* and *N. soroides* in the Tembat Reservoir are similar.

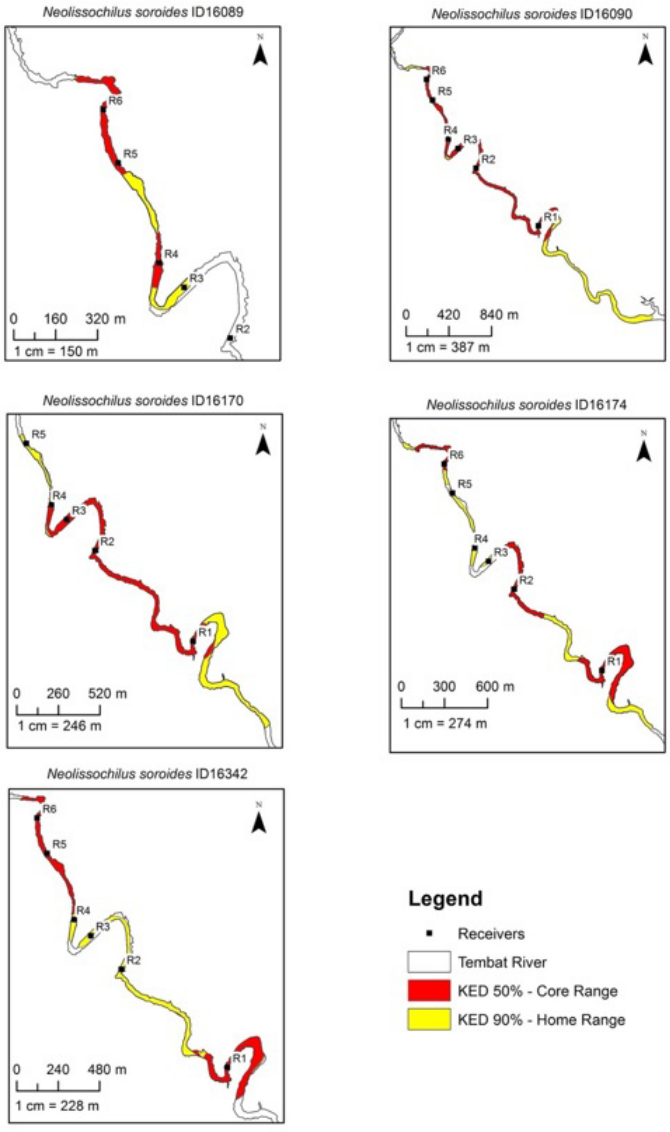

Figure 2. The kernel estimation density (KED) home range 90% (yellow) and core range 50% (red) for individuals of *Neolissochilus soroides* in Tembat Reservoir from April to December 2018

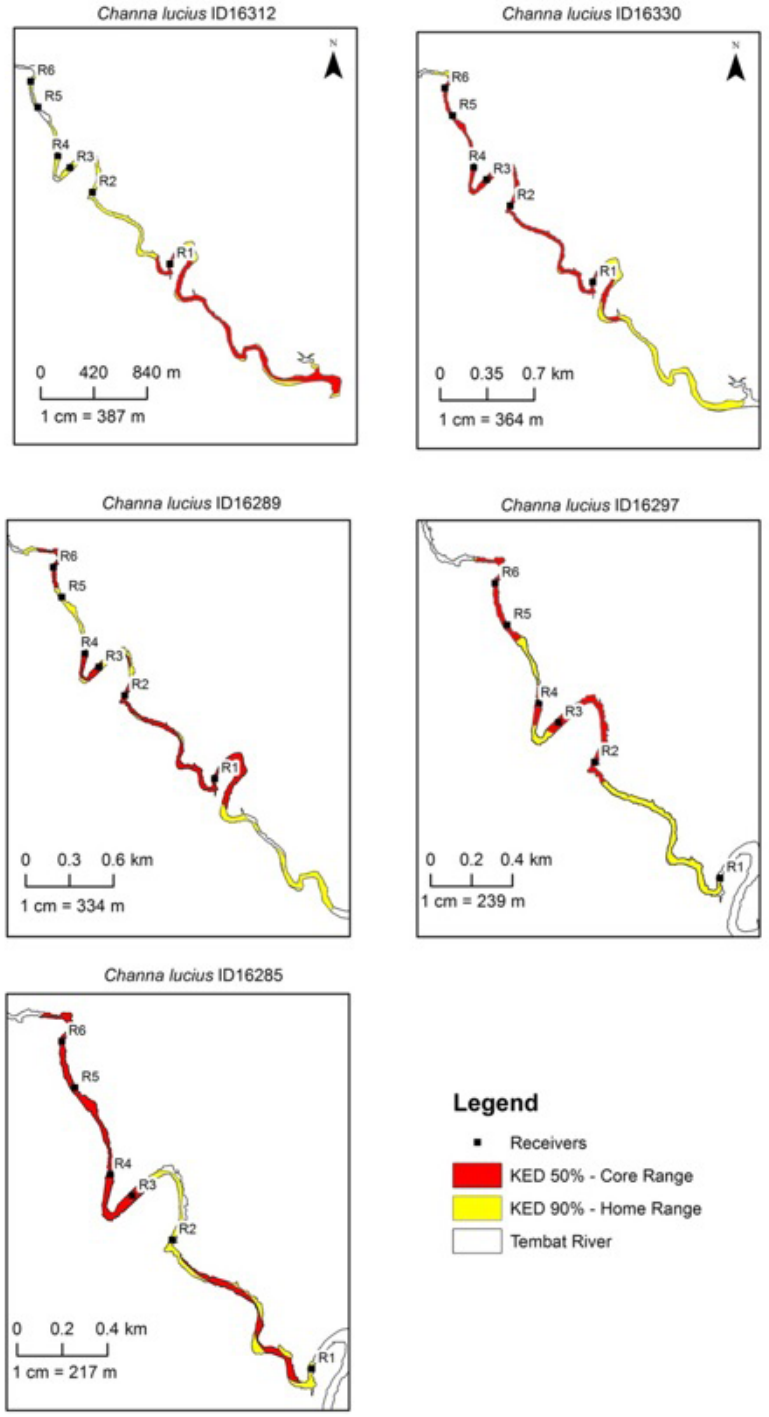

Figure 3. The kernel estimation density (KED) home range 90% (yellow) and core range 50% (red) for *Channa lucius* in Tembat River from April to December 2018

Table 2

The 50% KED (core area) and 90% KED (home range) by each of the individuals of both species in Tembat river from April to December 2018

Species	ID	TL (cm)	50% KED mean core area (m^2)	90% KED home range area (m^2)	No. of core area
Neolissochilus soroides	16089	40	7657.5 ± 3147.50	28078	2
Neolissochilus soroides	16090	35	4128.2 ± 1801.90	155848	10
Neolissochilus soroides	16170	37.5	4266.73 ± 3154.18	103964	11
Neolissochilus soroides	16174	43	4940.10 ± 2946.81	103725	10
Neolissochilus soroides	16342	41.5	6880 ± 3006.92	85822	6
Channa lucius	16285	43	71883	68399	1
Channa lucius	16289	43	10470.70 ± 9076.34	131992	7
Channa lucius	16297	41	12128 ± 4609.09	71883	3
Channa lucius	16312	43	25556.63 ± 21892.55	161425	3
Channa lucius	16330	53	12178.29 ± 8689.55	152974	7

Note. KED = Kernel estimation density

Time Spent in Various Zones of Tembat Reservoir

The average time spent by *N. soroides* and *C. lucius* at three different zones of the reservoir is shown in Figure 4. A one-way analysis of variance (ANOVA) conducted on the average time spent of *N. soroides* individuals resulted in a significant difference, $F(5,576) = 9.867$, $p < 0.001$. In which further Tukey test showed time spent in R2 (M = 2.71 hours, SE = 0.38) was significantly higher compared to R1 (M = 1.30 hours, SE = 0.13), R3 (M = 0.22 hours, SE = 0.09), R4 (M = 0.98 hours, SE = 0.27), R5 (M = 1.04 hours, SE = 0.23), and R6 (M = 0.46 hours, SE = 0.09). These results suggest that *N. soroides* spent more time at R2, which is at the transition zone of Tembat Reservoir. As for *C. lucius*, the average time spent between receivers locations also resulted in a significant difference, $F(5,360) = 12.489$, $p = 0.001$.

Tukey HSD test indicated that the average time spent at R5 (M = 7.28 hours, SE = 1.09) was significantly higher compared to the time spent at R1 (M = 1.78 hours, SE = 0.20), R2 (M = 1.95 hours, SE = 0.31), R3 (M = 2.16 hours, SE = 0.97), R4 (M = 1.55, SE = 0.76), and R6 (M = 3.46, SE = 0.61). Tukey test also reveals that the average time spent at R6 was significantly higher than R1 and R2. Specifically, this result suggests that *C. lucius* spent more time at R5 and R6, the riverine zone, compared to the lacustrine and transition zone of Tembat Reservoir. This result indicates a difference in response of both species towards the newly inundated area in Tembat reservoir where *C. lucius* was avoiding, and *N. soroides* was approaching the area.

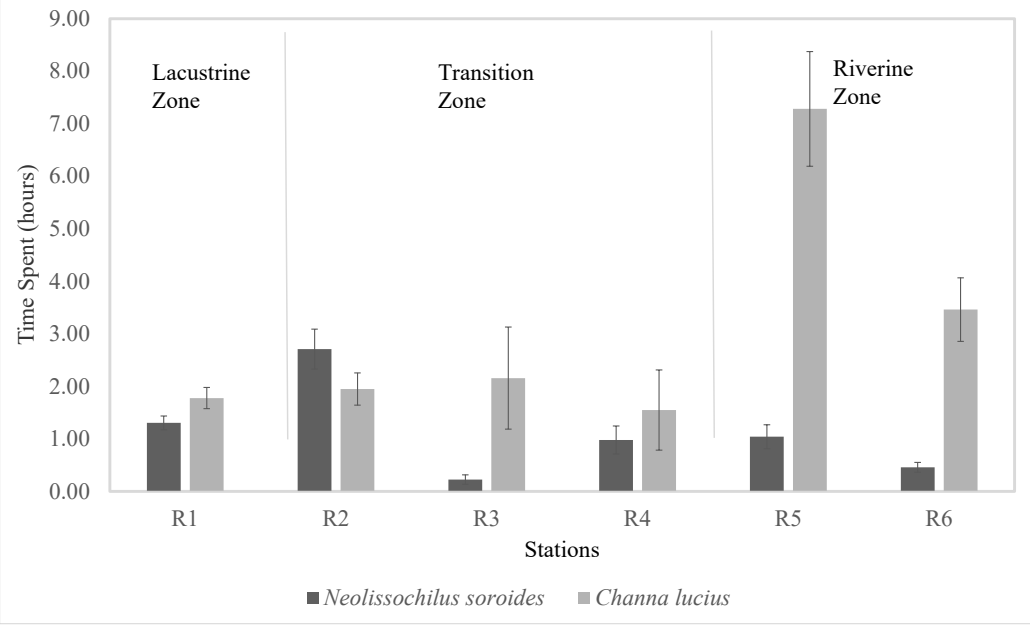

Figure 4. The time spent by *Neolissochilus soroides* and *Channa lucius* in the Tembat Reservoir system at different zones detected by the passive receivers (R1 – R6)

Habitat Parameters Associated with *Neolissochilus soroides* and *Channa lucius* in Tembat Reservoir

Based on the Kaiser-Guttman and broken stick model criteria, population components having eigenvalues greater than 1 represent shared variation and should be retained. For *N. soroides*, the first three components were identified to have eigenvalues greater than 1: PC1, PC2, and PC3, explaining 62.32% of the cumulative variance. PC1, PC2, and PC3 accounted for 29.70%, 18.03%, and 14.58% of the variance, respectively. Meanwhile, for *C. lucius*, PCA identified

the first four principal components to have an eigenvalue greater than 1. PC1, PC2, PC3, and PC4 jointly explained 71.19% of the cumulative variation. PC1, PC2, PC3, and PC4 accounted for 29.07%, 16.03%, 15.29%, and 10.80% of the variation, respectively (Table 3).

Table 3

The eigenvalue for each component and percentage of variance explained for Neolissochilus soroides *and* Channa lucius*, and bolded is the component retained*

Component	Eigenvalue	Variance (%)	Cumulative variance (%)
Neolissochilus soroides			
1	**2.97**	**29.7**	**29.7**
2	**1.8**	**18.03**	**47.73**
3	**1.46**	**14.58**	**62.32**
4	0.86	8.62	70.94
5	0.81	8.15	79.08
6	0.71	7.1	86.19
7	0.6	5.98	92.16
8	0.42	4.17	96.33
9	0.37	3.67	100
10	0	0	100
Channa lucius			
1	**2.91**	**29.07**	**29.07**
2	**1.6**	**16.03**	**45.1**
3	**1.53**	**15.29**	**60.39**
4	**1.08**	**10.8**	**71.19**
5	0.93	9.26	80.45
6	0.58	5.77	86.22
7	0.56	5.57	91.79
8	0.44	4.42	96.21
9	0.34	3.37	99.58
10	0.04	0.42	100

In this study, the strongest association in each component were considered by the highest covariance. The *N. soroides* factor loadings matrix showed that the first principal component had the strongest association with distance to bank and river width (r = 0.97). Meanwhile, the second principal component had a strong association with canopy cover (r = 0.77), and the third principal component was highly associated with substrate type (r = 0.79). The *C. lucius* factor loadings matrix

PCA indicated that the first principal component had the strongest association with mesohabitat type ($r = 0.79$). The second principal component indicated the strongest association with distance to riffle ($r = 0.97$). While the third principal component had the strongest association with the existence of canopy cover ($r = 0.82$), and the fourth principal component was strongly associated with elevation ($r = 0.90$) (Table 4).

Table 4

Loading matrix of the principal components with eigenvalue more than 1, Neolissochilus soroides *with 4 habitat parameters and* Channa lucius *with also 4 habitat parameters*

Habitat parameters	*Neolissochilus soroides*		
	PC 1	PC 2	PC 3
Distance to bank	0.97*	-0.03	0.11
River width	0.97*	-0.03	0.11
Distance to riffle	0.64	0.36	0.02
Canopy cover	0.01	0.77*	-0.08
Depth	0	0.66	-0.33
Submerge Large Wood (SLW)	0.02	0.64	0.46
Dist to lake	0.18	0.54	0.34
Mesohabitat	0.24	0.47	0.38
Substrate type	0.06	-0.07	0.79*
Bank type	0.07	0.06	0.73

Habitat parameters	*Channa lucius*			
	PC1	PC2	PC3	PC 4
Distance to bank	0.79*	0.08	0.02	-0.08
River width	0.74	0.18	-0.02	-0.43
Distance to riffle	0.70	0.17	-0.19	0.37
Canopy cover	0.55	0.2	0.24	-0.39
Depth	0.5	-0.17	-0.28	0
Submerge Large Wood (SLW)	0.05	0.97*	-0.03	-0.07
Dist to lake	-0.14	-0.96	0.11	0.05
Mesohabitat	-0.13	-0.05	0.82*	0.14
Substrate type	0	-0.09	0.80	-0.11
Bank type	-0.08	-0.06	0.06	0.90

Note. * Variable with the highest correlation with the principal component

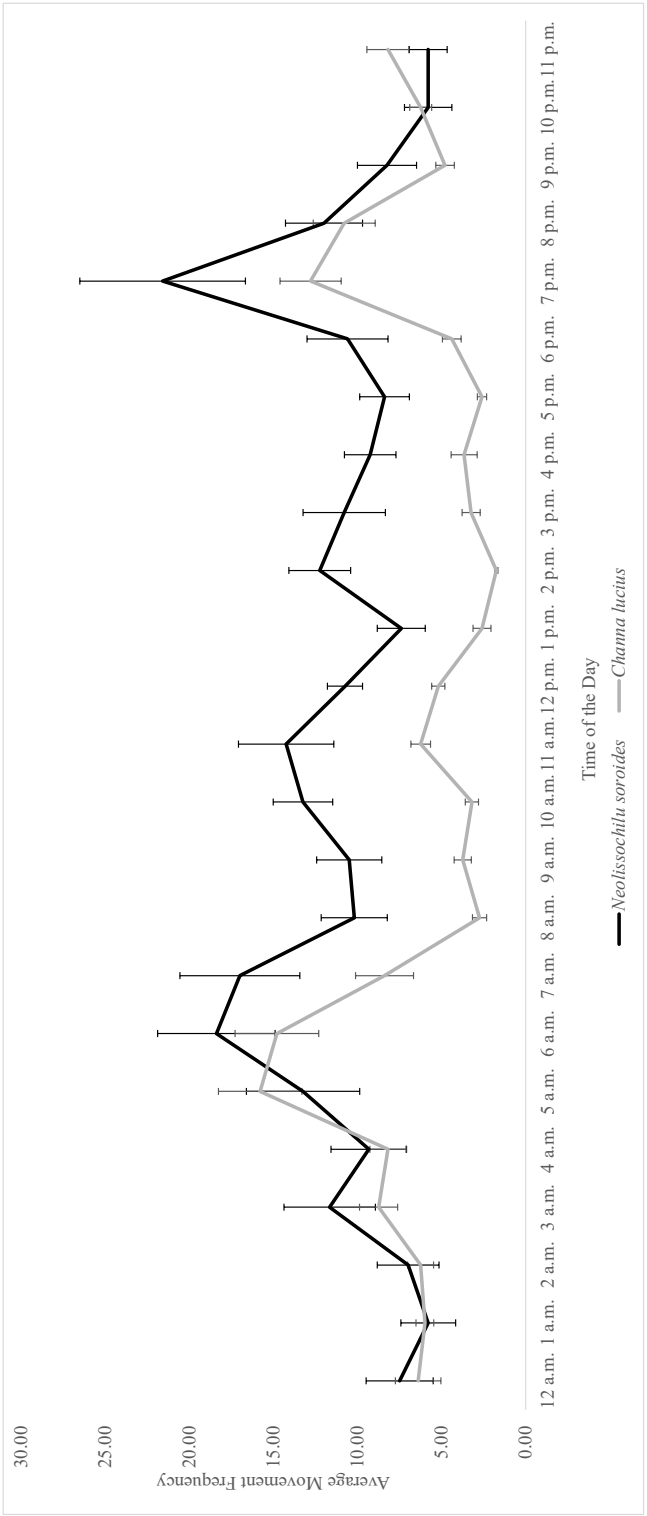

Figure 5. The hourly movement frequency by *Neolissochilus soroides* and *Channa lucius* between receivers in Tembat Reservoir from April to June 2018

Activity Pattern of *Neolissochilus soroides* and *Channa lucius*

The 24-hour average movement frequency of *N. soroides* and *C. lucius* is shown in Figure 5. Both species show high-frequency movement during dawn and dusk. Whereby, *N. soroides* showed two high peaks of movement frequency at 6 a.m. (M = 3.50, SE = 16.52), and 7 p.m. (M = 21.6, SE = 4.93). As for *C. lucius*, two high peaks of movement frequency were observed at 5 a.m. (mean = 15.8, SE = 2.48), and 7 p.m. (mean = 12.8, SE = 1.82).

No significant difference between daytime and nighttime movement for *N. soroides* were recorded. However, for *C. lucius*, three out of five individuals had significant differences between their daytime and nighttime movement: ID 16330 [t(32.658) = 12, $p = 0.021$], ID 16297 [t(-2.539) = 12, $p = 0.036$] and ID 16289 [t(-2.829) = 12, $p = 0.015$], which may indicate that this species make long-distance movements during night time.

DISCUSSION

Home and Core Range of *Neolissochilus soroides* and *Channa lucius*

The home range is commonly defined as where animals move from day to day (Hayne, 1949). This study defined the home range as to where the fish was detected and where it travels daily. In addition, the core area is defined to emphasise sections in a home range where the animal spends the greatest amount of time (Vander Wal & Rodgers, 2012). This study is the first study to document the movement pattern and home range of *N. soroides* and *C. lucius* in the freshwater environment in Malaysia using the acoustic telemetry method.

In this study, the home range size of *N. soroides* and *C. lucius* were similar, indicating that both species shared the same pathway. It is highly contributed to the morphology of the Tembat River that does not have many tributaries or small streams (TNBR, 2007). The river system runs on a nearly straight path and is not a complex river system compared to the neighbouring Puah catchment river system. Tembat River system is river order 3 and can be considered a small system that runs 45 km from the headwater (TNBR, 2007). Despite the similar average home range size between *N. soroides* and *C.lucius*, individual ID 16089 (*N. soroides*) had the smallest home range size (27.79 m^2) compared to other individuals. This individual was never found in the lacustrine zone and was recorded by R3, R4, R5, R6, and the upper stream of the R6 area. Comparing to the congeners, Lapointe et al. (2013) recorded the *Channa argus* home range in the Potomac River, USA, having approximately 60 times larger than *C. lucius* in this study. According to Woolnough et al. (2009), the size of the river system and the size of the fish influence the home range estimates regardless of linear type or areal type of home range.

As for *N. soroides*, a comparable species would be of the mahseer species. *Neolissochilus soroides* to coexist with *Tor* species in the Tembat River and Tiang River (Mohamad et al., 2020; Pinder et al., 2019; Sharir et al., 2019). A recent study on the

migration of *Tor putitora* and *Neolissochilus haxaganolepis* were conducted in Bhutan [Fisheries Conservation Foundation (FCF), 2019] recorded a larger home range size (more than 40 km distance) for these species compared to the findings in this study. Compared to its congener, a smaller home range of *N. soroides* and *C. lucius* reflects the smaller river system (Lonzarich et al., 2000). Larger systems are lesser geomorphic confining, thus allowing larger movement to be made as opposed to *N. soroides* in Tembat (Lonzarich et al., 2000).

Although there is no significant difference in the core area sizes, the variation in sizes observed cumulatively in the core area suggests that every individual movement is different in the ability or motivation to return to an area after moving throughout the river (Crook, 2004). A larger core area may indicate a territorial or predatory behaviour exhibited sporadically throughout the reservoir system (Figure 2 and Figure 3). The area highlighted in red is likely to be essential for each species, respectively. This area should be closely monitored to determine the importance of *N. soroides* and *C. lucius*. Food availability and predation risk may affect the motivation for an individual to have a different location of core area within the Tembat reservoir (Crook, 2004).

Time Spent in Various Zones of Tembat Reservoir

Channa lucius spent more time in the riverine zone, where the lotic system still influenced the area, and habitat heterogeneity is well preserved. This area provides sources of water inflow for the Tembat Reservoir and is not influenced by the outflow or dam operation. Mesohabitat characteristics such as pools and riffles are still present within the riverine zone. Furthermore, the existence of a fish sanctuary developed by Tenaga Nasional Berhad Research (TNBR) at the R5 (within the riverine zone) may be the reason for the significantly longer time spent by *C. lucius* in this area (Mohamad et al., 2020). *Channa lucius* is known as an ambush predator, which preys upon juvenile fishes abundance at R5. R5 provides a food source for *C. lucius* and shelter as the area was abundant with submerging large wood.

Neolissochilus soroides spent more time in the transition zone, converted into a nearly lentic system. The inundation of the Tembat River has created a trophic surge from the decomposed terrestrial vegetation, which usually increases the abundance and biomass of organisms utilising available resources (Arantes et al., 2019). These conditions may have become favourable to forage, and the availability of refuge may explain the significant amount of time spent in the area by *N. soroides*. The average depth in the transition zone, where *N. soroides* was found, was within the depth range where dissolved oxygen was still available (littoral zone). The littoral environment is known to have the highest biodiversity due to greater availability and heterogeneity of feeding resources, shelter, and habitat (Agostinho et al., 2008).

The age of the reservoir may become a significant factor for the utilisation by *N. soroides* and others ichthyofauna at the

transition zone of the Tembat reservoir, as it is spatially and temporally dynamic (Tian et al., 2020). The freshwater fish community is more influenced by the type of mesohabitat structure available in the river than catchment-scale processes, such as the logging history of the adjacent area (Wilkinson et al., 2018). However, the period since the logging activity correlates positively to the abundance of common cyprinids (Martin-Smith, 1998). Therefore, nutrient and food resources will likely deplete, and trophic structure collapse is predicted. A future survey in the transition and lacustrine zone should be conducted to observe the reservoir's impact and put mitigation measures into action.

Habitat Parameters Associated with *Neolissochilus soroides* and *Channa lucius* in Tembat Reservoir

Four habitat parameters were highly associated with *N. soroides* and *C. lucius* detection in Tembat Reservoir. However, only one parameter was similar between the species: the existence of canopy cover. Certainly, canopy cover protects fish from the glaring sun, maintain water temperature, and refuge from areal predators, such as birds of prey (Ceschin et al., 2015; Rutherford et al., 1997). However, it is noteworthy that most of the vegetation fringing the river in the Tembat catchment was wiped out during logging activity before the construction phase. As a result, the remains of riparian vegetation were scarce, but it remained the habitat that both of the species were detected. Thus, indicating the crucial role of riparian vegetation for the ichthyofauna in Tembat Reservoir.

Mesohabitat type was an important habitat characteristic in the detection of *C. lucius* in Tembat Reservoir. This species was commonly detected in the river run during the active daytime tracking. The river in the area studied was characterised by submerging large woods from the logging activity during the construction phase. It is assumed that *C. lucius* benefited from these structures for camouflage and shelter during the day. Several reports from this region also recorded a similar finding of *C. lucius* in submerged woody plants and aquatic vegetation area (Azrita, 2010; Rainboth, 1996).

Distance to riffle was one of the important habitat characteristics identified by PCA for *C. lucius*. This species was detected at an average of 846.14 m ± 95.43 m from the nearest riffle. Comparing this finding with *N. soroides*, which were detected at an average of 630.70 m ± 64.30m, *C. lucius* showed preferences further from faster-moving water. This is supported by the predatory behaviour of *Channa* species that have been recorded to habituate slow-moving rivers (Rainboth, 1996). Elevation was also identified by PCA to be highly associated with *C. lucius* detection in component 4. However, this parameter was the least concern in this study area because the range of elevation was not much of a difference, which was supported by the indifference velocity at all locations detected (data unpublished).

As for *N. soroides*, distance to bank and river width was the most important characteristics identified by PCA. These two parameters can be perceived as the size of the river because the distance to a bank can not exceed the river width. The size of the river plays a vital role in the detection of *N. soroides* and generally mahseer species. *Neolissochilus soroides* and other mahseer species are commonly found in the upper part of a river where the size of the river is relatively small (15-35 m wide) (Ambak et al., 2012; Khaironizam, 2010; Walton et al., 2017). In addition, the upper river supports the morphology and the physiological needs of mahseer, such as cooler water temperature, a more heterogeneous habitat (existence of pools, riffles, and runs), and food sources, such as algae and mayflies on the coarse river bed. Finally, substrate type was identified as one of the vital habitat parameters for *N. soroides*. During this study, *N. soroides* were commonly found on substrate type sand. It was not surprising as most of the river beds of Tembat River were smothered by sand and siltation due to the previous land clearing at the riparian area. The changes of river bed substrate are predicted to alter the foraging behaviour for not only *N. soroides* but other freshwater species, including invertebrates. Mahseer species are also known to spawn at the upper river with a coarse substrate such as cobbles and pebbles.

The type of habitat parameters associated with these two species can be a key habitat factor in establishing or identifying an area to be protected. For example, an area with submerged large wood should be protected by not removing or extracting the structure from the river. This area provides protection but also create the dynamic of hydrology that is essential for fish (Baillie et al., 2013). Based on this finding, removing riparian vegetation is not encouraged, and replanting of this area is needed.

Activity Pattern of *Neolissochilus soroides* and *Channa lucius*

Channa lucius exhibited nocturnal characteristics based on the movement behaviour detected by VR2W receivers deployed. During nighttime observation in Tembat Reservoir, *C. lucius* was often seen at the fringe of the river during dusk, in which it was hunting for juvenile fishes. Similarly, Horký et al. (2008) reported that *Sander lucioperca*, a European predatory freshwater species, were also found actively hunting for food at night. *Channa lucius* activity in the Tembat Reservoir appeared to be in line with its congener and other facultative air-breathing species that appear to be active at night (Boujard, 1995; Guo et al., 2017). Although *N. soroides* exhibit two-movement activity peaks during nighttime, they were not considered a nocturnal species. There were no significant differences in the individual average nighttime and daytime movement. These findings are supported by studies in Khaironizam (2010) that show *N. soroides* diurnally active and foraged during daylight based on the findings of the gut content. The movement recorded during the survey was the movement of the individual from one receiver to another receiver.

Therefore, it was considered a long-distance movement, as the passive trackers were deployed at least 200 meters from each other. Long-distance movement during the daytime may increase the risk of predation for fish by other species, such as birds, otters, and large fish (Shoji et al., 2017). This situation could explain the tendency in both species to make long-distance movements during nighttime to avoid predation. Time of day is one of the many factors that influence the movement of a fish for it to meet its metabolic or sheltering needs (Albanese et al., 2004). Many classical studies on the diel activity pattern of fish were associated with observations of feeding behaviour, as feeding is one of the main motivations of the fish to move from one place to another (Boujard, 1995; Clough & Ladle, 1997; Khaironizam, 2010; Weliange et al., 2006).

CONCLUSION

Channa lucius and *Neolissochilus soroides* utilise the same areas throughout the zones of Tembat Reservoir, which is most likely due to the non-complex river system. However, the zones in which the species prefer to stay longer were different, whereby *N. soroides* in the transition zone and *C. lucius* in the riverine zone may be motivated by the availability of specific food resources for each species. *Neolissochilus soroides* detection in Tembat Reservoir was highly associated with the size of the river, and the availability of canopy, cover and riverbed substrate, while *C. lucius* were four mesohabitat types, the distance to riffle, the availability of canopy cover and elevation. These key habitat parameters identified should be applied in the conservation management of an altered river in Tembat Reservoir. The 24-hour movement activity pattern recorded two peak hours during dusk and dawn for both species, further investigating for foraging and predator avoidance behaviour. Monitoring of habitat utilisation by fish fauna in the system should be conducted as the reservoir ages, especially in the inundated area where the ecosystem changes.

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Formulation and Antimicrobial Screening of *Piper sarmentosum* Cream against *Staphylococcus aureus*

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ABSTRACT

Piper sarmentosum, known as 'kaduk' has been traditionally used in different parts of the world to cure many diseases and ailments. It contains alkaloids and has been reported to possess many pharmacological properties like anti-cancer, anti-hyperglycaemic, anti-tuberculosis, antioxidant, and antimalarial. This study aimed to evaluate the formulation of *P. sarmentosum* cream and exploring the antimicrobial properties in different types of cream formulation before *in vivo* study. The leaves extract of *P. sarmentosum* was obtained from the cold-soaked methanolic extraction method, evaporated, and dried to produce the powdered extract. Then, it was diluted into four different concentrations, 25% w/v, 50% w/v, 75% w/v, and 100% w/v for *Staphylococcus aureus* antimicrobial screening. Based on the *S. aureus* antimicrobial screening, four types of creams were formulated (Cream A: cream base without *Piper sarmentosum* extract; Cream B: *Piper sarmentosum* extract (5%) only; Cream C: *Piper sarmentosum* extract (5%) with parabens preservatives; Cream D: *Piper sarmentosum* extract (5%) with vitamin E) and evaluated for their physical appearance, pH, stability study, and antimicrobial activity against *S. aureus*. As a result, 100% w/v concentration of the *P. sarmentosum* extract showed the highest result in the zone of inhibition (5.50 mm ± 0.03) towards *S. aureus* and was selected for cream formulation. In evaluating their physical appearance, all formulated creams showed high homogeneity and consistency with no phase separation and pH between 7.2 – 8.0 ± 0.07. On stability study, all creams with three different temperatures of 4°C, 27°C, and 37°C for 30 days show no colour

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changes, high homogeneity, and consistency with any phase separation. The results of antimicrobial screening for all formulated creams, show that Cream D has the highest zone of inhibition towards *S. aureus* (5.53 mm ± 0.25), followed by Cream C (4.43 mm ± 0.13). In conclusion, *P. sarmentosum* cream formulation showed high stability properties and possessed anti-microbial properties suggesting its potentials for wound healing cream.

Keywords: Cream formulation, methanolic extraction, *Piper sarmentosum*, *Staphylococcus aureus*

INTRODUCTION

Piper sarmentosum (*P. sarmentosum*), also known as 'kaduk' belongs to the Piperacea family. It is widely found in tropical countries in Southeast Asia, northeast India, and China (Karthigeyan et al., 2004). *Piper sarmentosum* has been used as a traditional remedy, in treating diabetes mellitus, cough, toothache, fungal infection on the skin, asthma, and inflammation of the pleura (Rahman et al., 2011). Chan and Wong (2014) reported that the leaves of *P. sarmentosum* contains phenylpropanoids, phenylpropanoyl amides, dihydro-flavones, and some essential oils. In addition, alkaloids isolated from *P. sarmentosum* leaves, 1-allyl-2, 6-dimethoxy-3 and 4-methylenedioxybenzene, have been claimed to show antibacterial activities against *Escherichia coli* and *Bacillus subtilis*.

The microorganisms commonly found in the wound infection are *Staphylococcus aureus* and beta-hemolytic *Streptococcus*, identified as a "transient flora" of the skin. Currently, *S. aureus* is overwhelmingly the most prominent cause of skin infections. This bacteria is usually harmless to human skin, but it can be infectious when there are injuries in the skin such as abrasions, cuts, surgical incisions wounds or indwelling catheters (Rain, 2005).

The cream, defined as a semi-solid emulsion, is applied to the skin. There are two types of cream: oil-in-water (o/w) cream and water-in-oil (w/o) cream. The oil-in-water emulsions are the most useful water-washable bases, while water-in-oil emulsions are used as an emollient and cleansing agent (Das et al., 2014).

Nowadays, the widespread belief that "green medicine" (plant-based products) are effectively safe to be used and less expensive. Therefore, the study of active compounds from natural resources has been actively pursued as it is essential in treating diseases (Ab Rahman et al., 2014). The situation prompts the concern in searching for natural compounds that could mimic the effects of synthetic cream but with fewer side effects. Although *P. sarmentosum* is commonly used as folk medicine, there is still little scientific investigation of its potential and preparation in being established as a treatment cream. Therefore, this study intends to evaluate the cream formulation and anti-microbial properties of formulated cream contain *P. sarmentosum* leaf extraction.

METHODS

Sources of *Piper sarmentosum*

The identified plant material was confirmed and validated by a botanist from Institute of Bioscience, Universiti Putra Malaysia (UPM), Serdang, Selangor. The voucher (SK 2100/15) has been deposited at the herbarium in UPM for future reference, while two kilograms of the leaf powder of *P. sarmentosum* was used for this study.

Preparation of *Piper sarmentosum* Leaves Methanolic Extract

The collected samples were washed with tap water and dried in the oven for 48 hours. The dried leaves were macerated to a fine powder before being soaked in methanol at 1:10 (Wong & Kitts, 2006). The mixture was then filtered and concentrated using rotavapor (Büchi®, Germany) to remove methanol. The concentrated extract was further dried using a freeze drier to remove residual methanol. Finally, the powder form of the extract was kept in vials with a tight cap before being stored in a –20°C refrigerator until further use (Fernandez et al., 2012).

In-vitro Antimicrobial Screening

In-vitro antimicrobial activity was examined for methanolic extractions and cream formulations. Prior to sensitivity testing, the *S. aureus* (ATCC11632) bacteria strains, Mueller Hinton (MH) agar (Pronadisa, Europe), and Mueller Hilton broth (Oxoid™, United Kingdom) were prepared. All the equipment used throughout the process was autoclaved at 120°C for 20 minutes to minimize contamination. Kirby-Bauer disc diffusion method was applied as an antimicrobial screening procedure. The bacteria strain *S. aureus* was cultured onto a blood agar plate and incubated at 37°C for 24 hours before conducting sensitivity testing. A single colony was then selected and cultured in 5 mL Mueller Hinton broth. The broth was adjusted to 0.5 McFarland standard using a UV-Vis spectrophotometer (Eppendorf BioSpectrometer®, Germany). Then, bacterial suspension turbidity was adjusted to the McFarland equivalence turbidity standard to produce bacterial counts in an expected range. The spectrophotometer was set at 600 nm or 625 nm, and sterile saline was used as the control.

Antimicrobial Screening of Local *Piper sarmentosum* Leaves Methanolic Extract

Piper sarmentosum methanolic extract powder was used to prepare four different concentrations of the extract. Purified distilled water was used to dissolve the powder extract to make the different concentrations of 25% w/v, 50% w/v, 75% w/v, and 100% w/v solution of the *P. sarmentosum* extract. Then, different solution concentrations were put into vials and kept at -4°C prior to use. Twenty (20) µL from each different concentration solution were used to impregnate a blank sterilized filter paper disc before being dried in a 37°C incubator for 24 hours prior to use for antimicrobial screening. Agar plates were inoculated with a standardized inoculum of

S. aureus strains. Then, filter paper discs (6 mm diameter), containing the test compound at the desired concentration, are placed on the agar surface. The Petri dishes are incubated at 37°C for 24 hours. Microbial growth was determined by measuring the diameter of the zone of inhibition of the extract using a transparent ruler in millimetres (mm).

Zone of inhibition (mm) = Diameter of microbial growth – Diameter of disc

Note. Diameter of disc is 6 mm

Preparation, Formulation, and Evaluation of *Piper sarmentosum* Cream Extract

From the antimicrobial screening results, the best extract concentration (100% w/v) that gave the highest resistance toward *Staphylococcus aureus* was chosen to be formulated into 60 g cream.

Oil in water creams was formulated based on the previous study (Gidwani et al., 2010). The extraction seeds of *Psoralea corylifolia* were substituted with *P. sarmentosum* extracts. First, oil in water cream without *P. sarmentosum* extracts was formulated, followed by the formulation of cream with *P. sarmentosum* extract without paraben preservatives. Then, creams with *P. sarmentosum* extract with paraben and vitamin E preservatives were formulated.

The formulation process was started by dissolving the oily and aqueous phase in a separate beaker in water bath at 75°C. The oil phase consisted of emulsifiers and other components and the extraction of *P. sarmentosum* (phase A). The aqueous phase was then dissolved, and water was added up to 100% of the overall formulation. Then, both phase A and phase B were heated up to 75°C using a water bath. After completing the heating process, it was followed by stirring the added oily phase into the aqueous phase until the mixture cools down. Then, the label formulated cream was transferred into a plastic container.

Evaluation of *Piper sarmentosum* Cream

In the antimicrobial test, as explained earlier, each cream has to undergo a visual inspection and pH test using a digital pH meter and rheological studies (Dahlan et al., 2014). All the formulated creams were evaluated for their colour, homogeneity and consistency, and phase separation. In addition, the appearance and presence of any aggregates were tested for all the formulated creams. The formulated creams' pH was measured three times using electronic pH meter (Mettler Toledo AG, United States of America). Stability studies were conducted for all types of the formulated creams. The evaluated parameters under stability studies include physical appearance and the pH of the cream. The stability studies were carried out at three different conditions with different temperatures, which are 4°C, 27°C, and 37°C for one month (International Conference of Harmonization [ICH] guidelines) (Dixon, 1998). The stability studies were conducted right after the completion of the formulation process.

Evaluation of Antimicrobial Properties of *Piper sarmentosum* Cream

The antimicrobial screening was conducted for the formulated cream to determine the ability of the cream to inhibit bacterial growth and to compare the activity of the extract in pure form and dosage form. The studies were carried out as same as the screening procedure. In addition, different types of cream were tested for antimicrobial activity by using the disc diffusion method. In this study, gentamicin antibiotics cream was used as the positive control. Meanwhile the negative control is the formulated cream without *P. sarmentosum* extract. The sensitivity of *S. aureus* strains towards *P. sarmentosum* extract cream was calculated by measuring the diameter (mm) of the zone of inhibition. The reading was taken at the end of 24 hours after incubation in a 37°C incubator. The plate with zone of inhibition (no growth around the disc) was sensitive to bacteria strain. The tests were conducted in triplicate for each formulation to ensure reproducibility. Appendix 1 shows the general formula based on two phases, which are oily and aqueous phases with different types of the formulated *P. sarmentosum* cream.

Statistical Analysis

Statistical analysis was performed using the Microsoft Excel 2007 and Statistic Package for Social Sciences version 25 (SPSS 25.0, 2017). ANOVA tests were used to identify the significant difference between groups followed by a post-hoc Tukey's test. The statistical significance was accepted when the *p*-value is less than 0.05.

Appendix 1
The general formula and different types of the formulated Piper sarmentosum *cream*

	General formula	Cream A	Cream B	Cream C	Cream D
Components	% w/w	Weight (g)			
Oily phase					
Stearic acid	2.5	1.500	1.500	1.500	1.500
White beeswax	1.5	0.900	0.900	0.900	0.900
Stearyl alcohol	5.0	3.000	3.000	3.000	3.000
Cetyl alcohol	6.5	3.900	3.900	3.900	3.900
Mineral oil	5.0	3.000	3.000	3.000	3.000
Aqueous phase					
Propylene glycol	5.0	3.000	3.000	3.000	3.000
Triethanolamine	2.0	1.200	1.200	1.200	1.200
Methyl paraben	0.01	0.006		0.006	
Propyl paraben	0.04	0.024		0.024	
Piper sarmentosum extract	5.0	3.00	3.00	3.00	3.00
Vitamin E	0.05				0.03
Water	Up to 100%				
Total (g)		60.00	60.00	60.00	60.00

RESULTS AND DISCUSSION

Evaluation of Antibacterial Properties of Local *Piper sarmentosum* Leaves Methanolic Extract

Four different concentrations (25% w/v, 50% w/v, 75%w/v, and 100% w/v) of the local *P. sarmentosum* leaves methanolic extract were tested against *S. aureus* using the Kirby-Bauer disc diffusion method. The antimicrobial activity of *P. sarmentosum* leaves extract could be seen through the average zone of inhibition of bacterial growth around the disc exhibited by various concentrations of *P. sarmentosum* extract against *S. aureus* strain. Figure 1 shows the average zone of inhibition exhibited by various concentrations of *P. sarmentosum* extract against *S. aureus*.

Figure 1 shows the zone of inhibition exhibited by various concentrations of methanolic extract of local *P. sarmentosum* leaves against *S. aureus*. There were significantly (p<0.05) lower inhibition of all the concentrations of *P. sarmentosum* extract discs against *S. aureus* compared with gentamicin antibiotic disc. Even though an increasing trend of the zone of inhibition could be seen between all the extraction concentrations, they are no significant differences between the groups. The minimum antimicrobial activity was exhibited by 25% w/v, while the highest was exhibited by 100% w/v concentration of *P. sarmentosum* extract. Figure 1 also shows that the sterilized distilled water does not affect the antimicrobial activity against *S. aureus*.

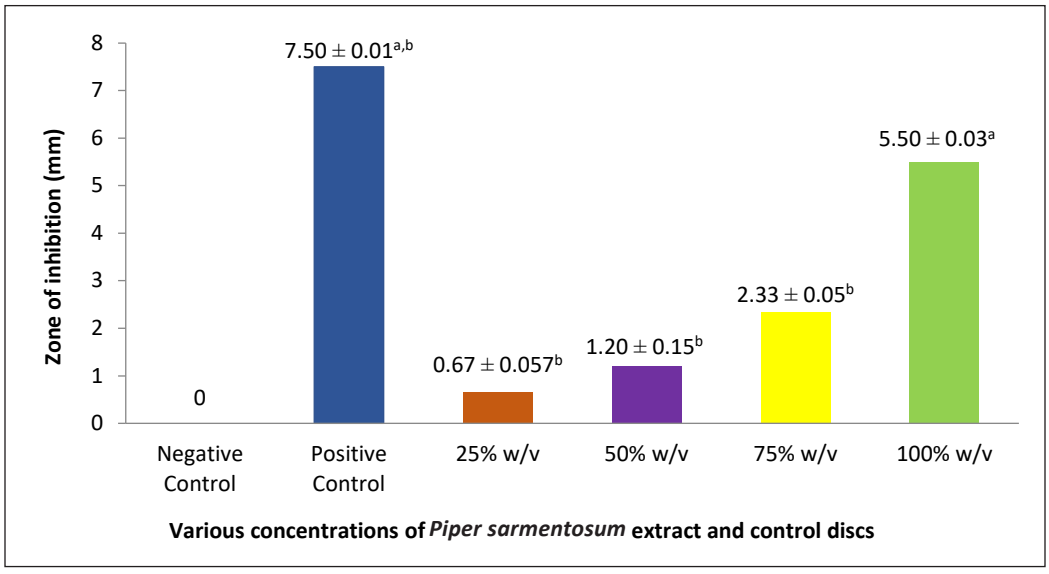

Figure 1. Values of zone of inhibition in sterilized distilled water discs (negative control), gentamicin antibiotic discs (positive control), 25% w/v, 50% w/v, 75% w/v, and 100% w/v concentrations of *Piper sarmentosum* extract discs against *Staphylococcus aureus* (n = 3). Each bar represents the mean ± SEM of the zone of inhibition

Note. [a]significant compared with the negative control while [b]significant compared with the positive control group (p<0.05)

Preparation, Formulation, and Evaluation of Local *Piper sarmentosum* Leaves Extract Cream

Based on the highest antimicrobial activity exhibited against *S. aureus*, the cream was formulated by incorporating 100% w/v of methanolic extract of *P. sarmentosum* leaves. These active ingredients were chosen due to higher inhibition against microbial growth that influenced the wound healing process. Different types of formulated cream were listed in Table 1.

Table 1
Type of creams and name for each cream

No.	Formulation	Name for each cream
1	Cream without *Piper sarmentosum* extract with parabens preservatives	Cream A
2	Cream *Piper sarmentosum* extract without parabens preservatives	Cream B
3	Cream *Piper sarmentosum* extract with parabens preservatives	Cream C
4	Cream *Piper sarmentosum* extract with vitamin E preservatives	Cream D

Evaluation of *Piper sarmentosum* Cream

Piper sarmentosum creams were evaluated based on the colour, homogeneity, consistency, and phase separation. Creams with *P. sarmentosum* extraction has dark green colour. All the formulated creams showed slightly different colour intensity and presented as homogenous semi-solid preparation shown in Table 2.

The pH formulations creams appear to become more alkaline across the concentration gradient, shown in Table 3.

Table 4 shows the condition of the creams after a month stored at different temperatures. There were no changes in colour, homogeneity, consistency, and phase separation of different creams with different temperatures (4°C, 27°C, and 37°C).

There was a slight change in the temperature of the cream within one month. Table 5 exhibits the pH's depreciation percentages of the formulated cream compared to the initial pH.

Evaluation of Antimicrobial Properties of *Piper sarmentosum* Creams

Different types of the formulated cream were tested using the antimicrobial activity against *S. aureus*. This study used the marketed dermatological semi-solid dosage of Diprogenta cream (gentamicin antibiotic cream) as a positive control. Therefore, it is important to compare the effectiveness of the formulated creams against a marketed product. Figure 2 shows the mean zones of inhibition exhibited by the different types of creams.

The methanolic extract was used in this study to obtain the pure extract of *P. sarmentosum* leaves. The methanolic extract method was chosen in this study because this method has been proven by Obeidat et al. (2012) to provide antimicrobial activity expression of the plant's extract. The efficacy of the plant extraction process was dependent on the solvent of extraction. Nayak et al. (2009) reported that both methanol and acetone were proven to be

Table 2
Physical appearance evaluation of Piper sarmentosum *cream*

Formulation	Colour	Homogeneity	Consistency	Phase separation
Cream A 	Whitish creamy	Good	Good	No
Cream B 	Light green	Good	Good	No
Cream C 	Green	Good	Good	No
Cream D 	Dark green	Good	Good	No

strong solvents in extracting inhibitory substances from medicinal plants. Lattanzio et al. (2006) also described that the phenolic and flavonoid compounds present in the extracts from various medicinal plants possess antimicrobial activity. Fernandez et al. (2012) presented their findings on *P. sarmentosum* leaves extraction with various phytochemicals except for saponins. Flavoinds and alkaloids are secondary metabolites classified as chemical classes that are generally activated with antimicrobial activities and soluble in polar solvents.

Table 3
The mean pH of the prepared cream formulations

Formulation	pH (Mean ± SEM)
Cream A	7.28 ± 0.01
Cream B	7.93 ± 0.01
Cream C	7.87 ± 0.01
Cream D	8.01 ± 0.07

Based on a preliminary investigation on antibacterial properties of methanolic extract of local *P. sarmentosum* leaves, 100% w/v of *P. sarmentosum* leaves extract was incorporated into a cream formulation. The cream is chosen as the semi-solid

Table 4
Condition of the various type formulated creams

Formulation	Temperature	Colour	Homogeneity	Consistency	Phase separation
Cream A	4°C	White creamy	Good	Good	No
	27°C	White creamy	Good	Good	No
	37°C	White creamy	Good	Good	No
Cream B	4°C	Light green	Good	Good	No
	27°C	Light green	Good	Good	No
	37°C	Light green	Good	Good	No
Cream C	4°C	Creamy dark green	Good	Good	No
	27°C	Creamy dark green	Good	Good	No
	37°C	Creamy dark green	Good	Good	No
Cream D	4°C	Creamy dark green	Good	Good	No
	27°C	Creamy dark green	Good	Good	No
	37°C	Creamy dark green	Good	Good	No

Table 5
Mean initial and final pH ± SEM and depreciation percentage after a month stored at different temperatures

Formulation	Initial pH	4°C	27°C	37°C
Cream A	7.28 ± 0.01	7.28 ± 0.00	7.29 ± 0.00	7.28 ± 0.01
		0.00%	0.14%	0.00%
Cream B	7.93 ± 0.01	7.87 ± 0.02	7.86 ± 0.01	7.92 ± 0.01
		0.76%	0.88%	0.13%
Cream C	7.87 ± 0.01	7.86 ± 0.01	7.87 ± 0.01	7.86 ± 0.05
		0.13%	0.00%	0.13%
Cream D	8.01 ± 0.01	7.95 ± 0.01	7.93 ± 0.01	7.94 ± 0.03
		0.75%	1.00%	0.90%

dosage in this study because it is easy to apply and well-absorbed by the skin. The formulation's vehicle is crucial in selecting the dosage as it will affect the delivery rate of the active pharmaceutical ingredients and the efficacy of the dosage. The active ingredient in creams is dissolving typically in an oil and water emulsion. The proportions of oil and water are approximately equal. Therefore, the creams are suitable for moistening the skin or exudative skin lesions (Chanwitheesuk et al., 2007).

The pH test of each formulated creams showed pH ranging from 7.0 to 8.0. In order to prevent or reduce skin irritation of topical and transdermal systems, it is important to maintain the pH of the topical formulations near the skin pH (Paudel et al., 2010). Besides that, it is also important to maintain the skin barrier function and defend against infections and diseases (Schmid-Wendtner & Korting, 2006). Cream with *P. sarmentosum* extract was evaluated for its stability at three different temperatures of

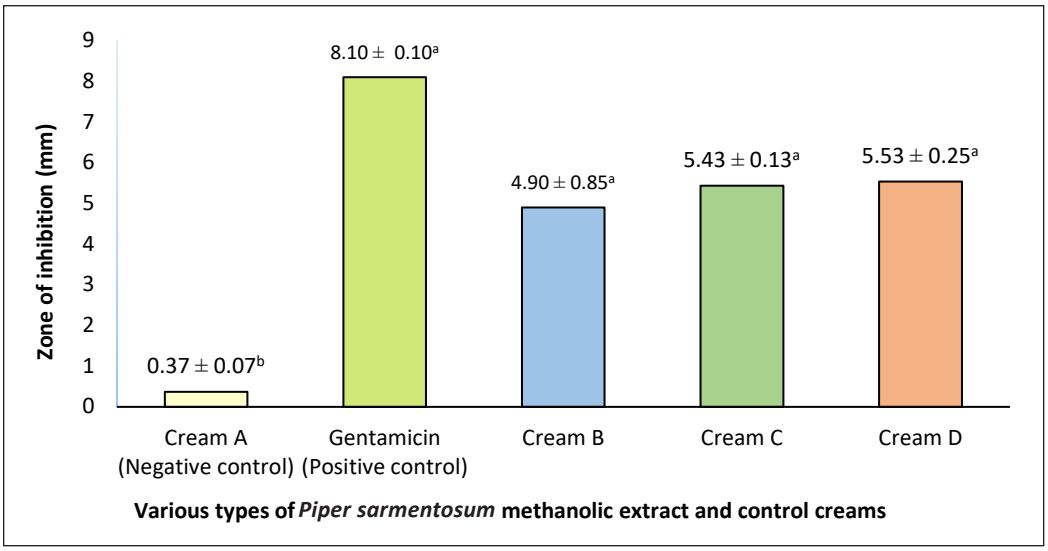

Figure 2. The zone of inhibition of the cream formulated with *Piper sarmentosum* methanolic extracts (Cream B: 4.90 ± 0.85; Cream C: 5.43 ± 0.13, and Cream D: 5.53 ± 0.25) were compared to the positive control cream (Gentamicin: 8.10 ± 0.10) and the negative control cream (Cream A: 0.37 ± 0.07), respectively. Each bar represents the mean ± SEM of the zone of inhibition

Note. [a]significant compared with the negative control while [b]significant compared with the positive control group (p<0.05)

4°C, 27°C, and 37°C for 30 days. Moreover, all the formulations are almost consistent in terms of their pH, colour, homogeneity, consistency, and phase separation even after a prolonged storage period at three different conditions (4°C, 27°C, and 37°C). All these tests are components of stability testing. The pH levels of the creams were in the range of 7.00 to 8.00 even after 30 days of stability testing. There were no colour changes while the creams' homogeneity and consistency remained stable (physical evaluation after formulation). The pH depreciation percentage of all creams showed less than 1.00% when compared to the initial pH. The results showed no indication of instability either in discolouration or a noticeable change in the formulated creams. The stability study passes the criteria stated in the United State Pharmacopeia (USP) for Pharmaceutical Compounding-Non-Sterile Preparation (Allen, 2012).

In this study, all the formulated creams containing *P. sarmentosum* extract showed growth inhibition towards *S. aureus*. In contrast, Cream A (without *P. sarmentosum* extract), which acts as a negative control, shows no zone inhibition towards *S. aureus*. Cream C (cream *P. sarmentosum* extract with paraben preservative) showed a higher average zone of inhibition when compared to Cream B (cream *P. sarmentosum* extract without preservative) and Cream D (cream *P. sarmentosum* extract with vitamin E as preservatives). Meanwhile, marketed gentamicin antibiotic cream, which acts as a positive control, showed the highest average zone of inhibition towards tested bacteria

strains. Based on a previous study conducted by Sahu et al. (2016), the preliminary investigation on the antimicrobial activity of *P. sarmentosum* showed that the methanolic extract of *P. sarmentosum* have the highest zone of inhibition towards Gram-positive bacteria (*S. aureus* and methicillin-resistant *Staphylococcus aureus* [MRSA]). Fernandez et al. (2012) indicated that the presence of flavonoids and alkaloids in the crude extract of *P. sarmentosum* is responsible for its antimicrobial activity.

The presence of secondary metabolites in the *P. sarmentosum* leaves extract may contribute to antimicrobial properties. Phenolics and flavonoids present in medicinal plants possess antimicrobial activity. The leaves of *P. sarmentosum* showed tannic acid, gallic acid, and quercetin in phenolics and flavonoids analysis. These compounds showed a mechanism of action towards the antibacterial activity of *P. sarmentosum* leaves. The mechanism of action, such as cytoplasmic membrane function, nucleic acids, and energy metabolism, acts by antimicrobial activities of naringin, quercetin, and rutin against human pathogenic microbes. A study by Syed et al. (2016) showed gallic acid and naringin in *P. sarmentosum* leaves in their antimicrobial activity.

In this study, a gentamicin antibiotic disc was used as the positive control, which exhibited higher antimicrobial activity against *S. aureus* strain with a larger zone of inhibition as compared to 100% w/v extract of *P. sarmentosum* leaves ($p<0.05$). Gentamicin antibiotic is an established antimicrobial agent used in clinical settings. Thus, it showed remarkable antimicrobial properties in this study. This conventional antibiotic was used as the positive control because of its higher inhibition of bacterial growth. Various established researches and lab works have been carried out to test the efficacy of the substances and drugs as antimicrobial agents. Gentamicin is an aminoglycoside antibiotic used to treat many types of bacterial infections, particularly those caused by Gram-negative organisms, including *Pseudomonas*, *Proteus*, and Gram-positive *Staphylococcus* (Ghashghaei & Emtiazi, 2013).

Fernandez et al. (2012) indicated that the presence of flavonoids and alkaloids in the crude extract of *P. sarmentosum* is responsible for its antimicrobial activity. The other three concentrations (25% w/v, 50% w/v, and 75% w/v) did not show a clear zone of inhibition of growth towards *S. aureus*. Based on a study conducted by Rain (2005), the antimicrobial activity of *P. sarmentosum* leaves showed a zone of inhibition against *S. aureus* (9 mm) with a concentration of 2000 µg/disc. This study did not mention the concentration of the extract before it was impregnated into the discs. Our findings were in line with a previous study by Fernandez et al. (2012), which stated that the antimicrobial activity of *P. sarmentosum* leaves would show better results at higher concentrations. Through our findings, concentrations less than 100% w/v may be insufficient to show antimicrobial activity. It is also important to note the possibility of contamination,

which may cause the inability of the three concentrations below 100% w/v (25% w/v, 50% w/v, and 75% w/v) to show clear zones of inhibition towards *S. aureus*.

The *P. sarmentosum* leaves could contribute to the antibacterial activity by the mechanical action of these compounds. Recent studies by Cushnie and Lamb (2005) found that antimicrobial activity of naringin, quercetin, and rutin against human pathogenic microbes showed mechanism actions of energy metabolisms, nucleic acid synthesis, and cytoplasmic membrane function. In addition, the leaf and fruit of *P. sarmentosum* were found to have gallic acid that contributes to the antibacterial activity of extraction against *S. aureus*. Paudel et al. (2010) reported that gallic acid exhibited potent activity against fungal and human pathogenic bacteria.

CONCLUSION

The present study found that *Piper sarmentosum* leaf methanolic extract is a potential antimicrobial agent as it showed antimicrobial properties against *Staphylococcus aureus* in the *in-vitro* antimicrobial assay. The cream formulation possesses anti-microbial properties showed a similar effect as in the antimicrobial screening of extraction. This study exhibited that the *P. sarmentosum* leaf could be safely used to treat in various diseases by increasing physical appearance, homogeneity, and consistency in stability studies. However, the previous study did not continue for further isolation of alkaloids. The same alkaloids are essential in exhibiting the significant antimicrobial properties of *P. sarmentosum* extract, which need further investigation in the future.

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Evaluation of Properties and Elements in the Surface of Acidic Soil in the Central Region of Thailand

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ABSTRACT

The study aimed to evaluate and correlate acidic soil components to understand the phenomena of this type of soil. The soil samples were collected from 64 locations in 3 provinces of central Thailand and were tested for soil pH, element content, soil organic matter (SOM), and soil organic carbon (SOC). The results show that soil acidity in central Thailand has an average pH of 4.71 ± 0.87. The soil acidity level ranges from very strongly acidic in Phatum Thani and Nakhon Nayok provinces to strongly acidic in Chachoengsao province. Soil bulk density is about 0.34 g/cm^3, and the correlation of soil pH to lead (Pb), nickel (Ni), nitrogen (N), carbon-to-nitrogen ratio (C/N ratio), and zinc (Zn) is as follows: principle component 1 (PC1) is carbon-to-nitrogen ratio > pH > zinc (C/N ratio > pH > Zn), and principle component 2 (PC2) is soil organic carbon > bulk density > soil organic matter (SOC > BD > SOM). Soil pH, SOM, and SOC are in similar groups. The soil abundance at the study site was compared with the ideal soil for plants, and heavy metal contamination in the acidic soil of the central region did not exceed the standard limit. The study found a correlation between SOM and SOM (r = 0.715; p < 0.01), indicating soil quality and microbial activity.

Keywords: Acidic soil, central region, soil component, soil property

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INTRODUCTION

Soil is the main factor for success in agricultural production (Amenyan, 1988). Highly fertile soil produces a good agricultural yield (Parikh & James, 2012), and soil with a pH below 5.5 will affect plant growth rate (Neina, 2019). Soil with a pH below 5.5 is considered acidic, so acidic soil covers about 3.95 million km^2, or 27% of the world's land surface (von Uexküll & Mutert, 1995). In Asia, acidic soil covers about 1.04 million km^2 or 26% of the total area of acidic soil in the world (Van Ranst et al., 2004). In Thailand, sulfur is present on the soil surface (depth > 5 cm) in about 8,904.5 km^2, or approximately 0.000086% of the Asian acidic soil area. It is distributed throughout the country's central region, representing 5,097.4 km^2, or 57.2% of the acidic soil in Thailand (Research and Development for Land Management Division, 2006).

The influence of soil acidity on various aspects of crop production, including growth, biomass allocation, nutrient uptake, mycorrhizal colonisation (Soti et al., 2015), and plant stress (Haling et al., 2011), is minimal. However, acidic soil may have a positive effect on the growth of some plants, such as tea and palm oil (Chien et al., 2019; Ho et al., 2019), and soil pH affects the mineralisation of organic material in the origin soil (parent soil) (Conyers et al., 1995).

Acidic soil covers the surface of the central region of Thailand in the irrigation zones and agricultural areas of Nonthaburi, Phathum Thani, Phanakhonsri Ayuthaya, Nakhon Nayok, and Chanchoengsao provinces. The water supply is essential to agricultural production in acidic soil zones because it affects the ion exchange between a liming material, such as calcium carbonate, and water, forming a hydroxyl group (OH^-) ion or the oxidation of organic sulfur (S) to sulfate ion (SO_4^{2-}) accompany by an equivalent quantity of hydrogen anion (H^+) (Freedman, 1995; Sparks, 2003). Without water, soil acidity will be a more significant factor in supporting agriculture in the area of bulk density (BD) when considering pores in the soil, soil organic matter (SOM), and soil organic carbon (SOC) along with organic fertility or soil biomass (Bautista et al., 2016) and multi-element. Therefore, it will also support agricultural production in acidic soil zones (Joris et al., 2013).

The objective of this study was to evaluate the relationship among acidic soil properties and analyse soil properties such as pH, percentage of wet material in the soil, soil electrical conductivity (EC), soil moisture, BD, SOM, and SOC in Thailand's central region. In addition, this study also looks at element compounds in acidic soil and considers the correlation of these soil components. This information is essential in understanding the physiochemical phenomena of acidic soil in this zone and the influence of elements on plant nutrients, including heavy metals (Fontes & Alleoni, 2006), so that techniques may be developed to improve future soil quality in the country.

MATERIALS AND METHODS

Study Sites

This study covers acid zones in 5 districts

of 3 provinces: (1) In Nong Suea district in Pathum Thani province, 21 soil samples were collected from 7 zones; (2) in Ongkharak and Banna districts in Nakhon Nayok province, 21 samples were collected from 7 zones; and (3) in Bangnampraw and Muang districts in Chachoengsao province, 22 samples were collected from 7 zones. The study site focused on the agriculture area on the acidic soil map provided by the Land Development Department of Thailand, as presented in Figure 1 and Table 1.

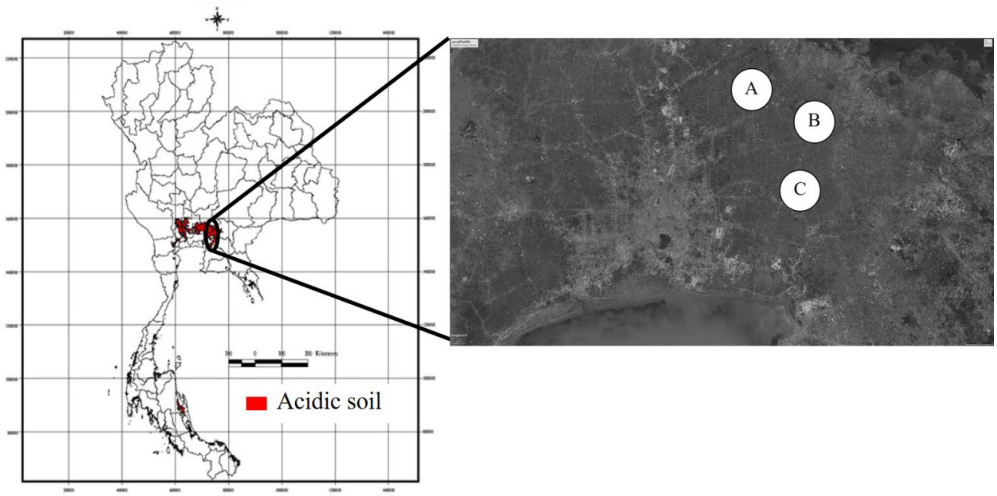


Figure 1. Study sites: (A) Nong Suea district in Pathum Thani province; (B) Ongkharak and Banna districts in Nakhon Nayok province; (C) Bangnampraw and Muang districts in Chachoengsao province

Table 1

Land use of study sites where soil samples were collected

Area	Land use							Total *N*
	1	2	3	4	5	6	7	
A	Banana farm	Lemon garden	Orchard	Papaya garden	Rubber plantation	Lemon garden	Cassava field	21
N	(3)	(3)	(3)	(3)	(3)	(3)	(3)	
B	Fallow	Paddy field	Backyard garden	Banana farm	Paddy field	Fallow	Abandoned	21
N	(3)	(3)	(3)	(3)	(3)	(3)	(3)	
C	Grass field	Lemon-grass field	Galangal field	Paddy field	Integrated farming	Paddy field	Coconut garden	22
N	(3)	(3)	(4)	(3)	(3)	(3)	(3)	
							Total samples	64

Note. *N* = sample collected from land classified as a farm zone

Soil Moisture, Soil Temperature, and Soil Electrical Conductivity

The moisture, temperature, and EC of soil collected from the surface (0–5 cm) and the data in the field survey on soil moisture—or percentage of wet soil (% Wet)—temperature, and EC were collected using series HH2 Delta-T Devices. These are automatic instruments for collecting data.

Soil pH Measurement and Validation

The soil pH meter used for collecting data in the field survey (*F.*pH, the pH value that collected from the field) was a soil tester manufactured by Takemura Electric Works LTD (Japan). The pH value of the samples was then tested using a solution technique. The soil sample from the field (soil surface 0-5 cm) was dissolved in water at a 1:1 ratio of 5 g of soil diluted in 5 mL of deionized water and then stirred for 30 min. After waiting an additional 30 min to allow for precipitation, the liquid was separated from the sample for pH checking (*L.*pH, the pH value of the laboratory test) using a series HQ40d Portable Multi Meter by Hach (United States of America). However, regarding the variation between the laboratory and the field (McKean & Brent, 1989; Neal & Thomas, 1985), Shaver (1993) reported an average pH variation of +0.132, as well as Lataysh and Gordon (2004) suggested that the pH value in the field will be greater than that in the laboratory by about 0.10 pH. In this report, the application of the soil pH value between fieldwork and laboratory analysis is derived from the following equation:

$$\mathrm{pH} = \frac{(F.pH + 0.1) + (L.pH - 0.1)}{2}$$

The 0.1 value is applied according to Latysh and Gordon's (2004) observation ratio for balancing pH value.

Soil Organic Carbon and Total Nitrogen

In the study, total levels of carbon and nitrogen in the soil were measured using the LECO series CHN-628 CHN Analyzer (United States of America). After collection, the percentage of carbon in the soil was calculated as SOM using a formula from the Soil Lecture Team (2006):

$$\% \text{ SOM} = 100\ (\% \text{ C/c}) \qquad [1]$$

where % SOM = Percentage of soil organic matter, % C = Value from CHN analyzer processing, and c = % Weight of SOC use 52 for topsoil calculated (Soil Lecture Team, 2006). % SOM will be used to calculate the weight of SOM content in the soil according to the formula:

$$\text{SOM (mg/kg)} = [\% \text{ SOM} \times (W1/100)] \times 10^6 \qquad [2]$$

where SOM = Soil organic matter (mg/kg), % SOM = Rate from the first equation, and W_1 = Dry weight of the sample (mg) in the experiment using 2 mg/sample.

However, SOC uses a soil sample from the topsoil at a depth of not more than 20 cm, so Han et al. (2018) and Y. Liao et al. (2015) suggest the following calculation:

$$SOC\ (mg/kg) = \%\ SOM \times 0.58 \times 100$$

where SOC (mg/kg) = Soil organic carbon concentration, % SOM is from formula [1], and 0.58 is the van Bemmelen conversion factor of 58% C in SOM.

Elements in the Soil Content

The soil was collected from 8 points (in a Z shape) in the field and mixed to create 1 sample (difference a soil to use find BD). The soil was dried in a hot 105°C oven for 3 days and then ground using a mortar and pestle. A net 10 mm of sifted soil was selected and maintained at a temperature of −4°C. The soil extraction used in atomic absorption spectrometry (AAS) analysis was a 2 g soil sample with concentrated nitric acid (HNO_3) and concentrated perchloric acid ($HClO_4$) in a 1:1 ratio for 10 mL (United States Environmental Protection Agency, 1996). It was then digested at about 500°C in the SpeedDigester K-425 BUCHI (Switzerland) until dried. Each residue was rinsed with 1% HNO_3, followed by sieving through Whatman No. 1 paper. The supernatant was then transferred to a 50 mL volumetric flask, and 1% HNO_3 was added for continued AAS analysis (Thummahitsakul et al., 2018).

The mineral analysis set the standard of lead (Pb), copper (Cu), cadmium (Cd), zinc (Zn), selenium (Se), iron (Fe), mercury (Hg), potassium (K), and nickel (Ni) prepared by a solution of an Agilent Technologies (United Sates of America) concentration of 1,000 µg/mL stock of each heavy metal and 1% HNO_3, with a linear standard calibration curve to measure mineral samples. The Pb, Cu, Cd, Zn, Se, Fe, Hg, K, and Ni analyzing by AAS, an Agilent series 240AA instrument (United Sates of America).

Whereas, phosphorus in the soil content was analysed using the Bray II method and measured by spectrophotometers in wavelength 882 (nm). Available phosphorus was in the form of potassium dihydrogen phosphate (KH_2PO_4) in soil samples.

Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA) for variances. Differences in data were compared by post-hoc Tukey's honestly significant difference (HSD) test in $p < 0.05$ between data components. Principal component analysis (PCA) evaluated correlation matrix components with factors of influence related to acidic soil in the central region. Correlation analysis considered the use of Pearson's correlation ($p < 0.05$). Finally, all analyses were conducted using the programs Statistical Package for the Social Sciences (SPSS) V.22 and SigmaPlot 12.0 (free trial).

RESULTS AND DISCUSSION

Soil Physical Properties of the Study Sites

In central Thailand, the soil pH ranged from 2.89–6.30, with an average of 4.71 (± 0.87). The soil pH of Chachoengsao province has a significantly ($p < 0.05$) to that of Pathum Thani and Nakhon Nayok provinces (Table 2). The acidic soil of Phatum Thani and Nakhon Nayok is classified as extremely acidic (pH between 3.5–4.4), and the soil of Chachoengsao is classified as strongly acidic (pH between 5.1–5.5), according

to the Natural Resources Conservation Service (NRCS) (1994). The BD averaged 0.34 ± 0.11 g/cm^3. Chachoengsao has a significantly ($p < 0.05$) lower BD (0.28 g/cm^3), while Phathum Thani (0.37 g/cm^3), but not significant to Nakhon Nayok provinces, have a BD of 0.35 g/cm^3. The result indicates that the areas are suitable for agriculture because of the characteristics of loam clay and because BD is related to soil texture and parent material. The % Wet in the soil averaged 36.9% (± 14.4). This high value can be attributed to the data being collected during the rainy season in Thailand. The EC averaged 205 µS (± 133), soil temperature averaged 32.14°C (± 2.90), and the soil moisture level averaged 19.12% (± 5.21), as shown in Table 2. The correlation of soil pH in relation to EC ($r = 0.274$, $p < 0.05$), BD in relation % Wet ($r = 0.354$, $p < 0.01$), and % Wet and EC ($r = 0.410$, $p < 0.01$) are presented in Table 3.

Table 2

Physical properties of acidic soil in central Thailand

	pH	Bulk density (g/cm^3)	Percentage of wet soil (% Wet)	Electrical conductivity (µS)	Temperature (°C)	Moisture (%)
Pathum Thani	4.41 ± 0.59^a	0.37 ± 0.06^a	41.8 ± 17.35^a	173 ± 66.4	33.9 ± 2.80^a	19.8 ± 4.01^b
Nakhon Nayok	4.18 ± 0.82^a	0.35 ± 0.08^{ab}	38.2 ± 8.69^{ab}	155 ± 61.6	29.8 ± 2.45^b	15.8 ± 6.62^a
Chachoengsao	5.50 ± 0.54^b	0.28 ± 0.15^b	31.1 ± 15.50^b	291 ± 192	32.6 ± 1.77^{ab}	21.5 ± 2.75^b
Average	4.71 ± 0.87	0.34 ± 0.11	36.9 ± 14.48	205 ± 133	32.1 ± 2.90	19.1 ± 5.21

Note. ^{a,b} The mean difference is significant at the p-value < 0.05 level (HSD)

Table 3

Correlation of physical properties of acidic soil in central Thailand (n = 64)

	pH	Bulk density (BD)	Percentage of wet soil (% Wet)	Electrical conductivity (µS)	Temperature (°C)
BD	−.030	-			
% Wet	−.151	.394**	-		
EC	.274*	.354**	.410**	-	
Temperature	−.048	−.085	−.070	.110	1
Moisture	.245	.055	.159	.241	.216

Note. *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

Relationship between SOC, SOM, and C/N ratio in Acidic Soil in Central Thailand

SOC is one indicator of soil quality and is related to microorganism activity. The SOC content of acidic soil in central Thailand averaged 404 ± 303 mg/kg, and the acidic soil level in Phatum Thani, Nakhon Nayok, and Chachoengsao was not significant ($p > 0.05$) (Table 4). The SOM averaged 9,036 ± 4,048 mg/kg, and the acidic soil level in Phatum Thani, Nakhon Nayok, and Chachoengsao was not significant ($p > 0.05$). The C/N ratio averaged 3.69 ± 2.13, significant ($p < 0.05$) in Chachoengsao to Phatum Thani and Nakhon Nayok. However, when considering the correlation between SOC, SOM, and the C/N ratio, it was found that SOM was related to SOC ($r = 0.715$; $p < 0.05$), and SOM was related to the C/N ratio ($r = 0.283$; $p < 0.05$), as seen in Table 5. The C/N ratio increased with the SOM value and decreased with the SOC rate, as presented in Figure 2.

Table 4

Average of SOC, SOM, and C/N ratio in acidic soil in central Thailand

Items	Phatum Thani	Nakhon Nayok	Chachoengsao	Average
SOC (mg/kg)	457 ± 251[a]	468 ± 405[a]	294 ± 201[a]	404 ± 303
SOM (mg/kg)	9866 ± 2627[a]	9615 ± 6045[a]	7690 ± 2207[a]	9036 ± 4048
C/N ratio	3.29 ± 1.22[a]	1.21 ± 0.89[a]	5.05 ± 2.04[b]	3.6 9 ± 2.13

Note. [a,b,c] The mean in row differences is significant at the *p*-value < 0.05 level (HSD); SOC = Soil organic carbon; SOM = Soil organic matter; C/N ratio = Carbon-to-nitrogen ratio

Table 5

Correlation of SOC, SOM, and C/N ratio in acidic soil in central Thailand

Item	SOC	SOM	C/N ratio
SOC		0.715**	0.118
SOM	0.715**		0.283*
C/N ratio	0.118	0.283*	

Note. *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed); SOC = Soil organic carbon; SOM = Soil organic matter; C/N ratio = Carbon-to-nitrogen ratio

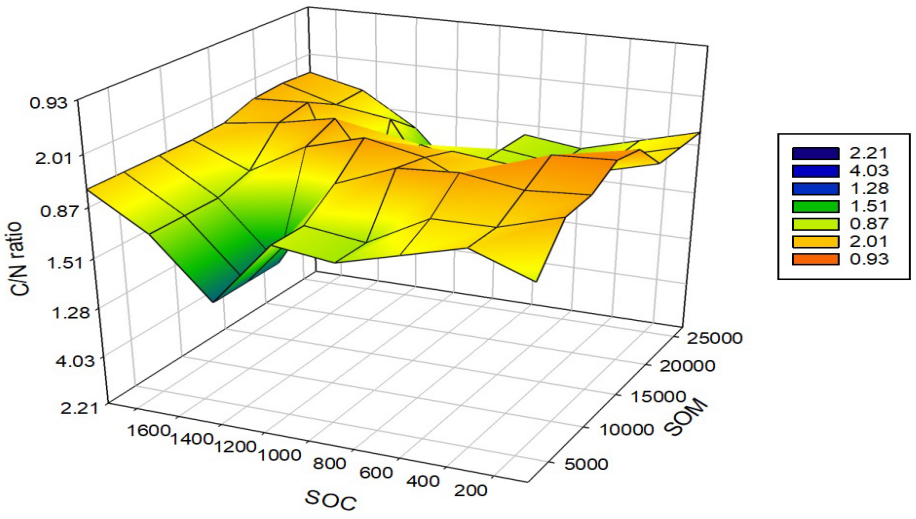


Figure 2. Evaluation rate of C/N ratio between SOC and SOM in acidic soil in central Thailand

Elements of Acidic Soil Content

The study of the mineral content of acidic soil in the central region found Fe present in the highest quantity. At the same time, Ni made up the smallest quantity, as presented in Table 6. Soil elements can be classified into three groups: essential macroelements, including N, P, and K; essential microelements, such as Fe, Cu, Zn, and Ni; and heavy metals, like Pb, Cd, Hg, and Se. The number of essential macroelements showed that N > P > K, and the quantity of N content in Nakhon Nayok > Phatum Thani > Chachoengsao was significant ($p < 0.05$). P levels in the soil content in Phatum Thani and Chachoengsao were significantly higher ($p < 0.05$) than those in Nakhon Nayok, and K levels found in the soil content in Phatum Thani and Chachoengsao were significant ($p < 0.05$), as presented in Table 7. The proportion of N, P, and K in acidic soil is a 13:2:1 ratio.

The number of essential plant microminerals in acidic soil content was Fe > Cu > Zn > Ni. The results showed that the quantity of Fe and Zn in Phatum Thani and Chachoengsao was significant ($p < 0.05$) compared to Nakhon Nayok province. Phatum Thani's Cu quantity was higher ($p < 0.05$) than that of Nakhon Nayok and Chachoengsao provinces, and the Ni quantity of the three provinces differed ($p < 0.05$), as shown in Table 7.

The quantity of the heavy metal Pb in Nakhon Nayok was higher than in Phatum Thani and Chachoengsao ($p < 0.05$), and the quantity of Hg found in Phatum Thani and Nakhon Nayok was not significant ($p < 0.05$) compared to Chachoengsao. However, differences in Cd and Se levels between the three provinces were small.

Table 6

Average total quantity of elements in acidic soil in the central region

Elements (mg/kg)	Average	SD	%
Pb	130	103	0.42
Cd	2.25	1.78	0.02
Hg	5.43	2.16	0.03
Se	1.83	0.70	0.01
Fe	29765	8921	96.2
Cu	40.56	40.2	0.13
Zn	13.35	12.2	0.04
Ni	0.19	0.04	0.00
N	782	377	2.53
P*	131	122	0.42
K	61	29	0.2
		Total	100

*P was determined using the Bray II method. The phosphorus considers P available from potassium dihydrogen phosphate (KH_2PO_4); Pb = Lead; Cd = Cadmium; Hg = Mercury; Se = Selenium; Fe = Iron; Cu = Copper; Zn = Zinc; Ni = Nickel; N = Nitrogen; P = Phosphorus; K = Potassium

Table 7

The quantity of elements for plants in acidic soil content classified by group and province

	Phatum Thani	Nakhon Nayok	Chachoengsao
Essential macro-elements (mg/kg)			
N	842 ± 237^{b}	$1{,}097 \pm 359^{a}$	425 ± 124^{c}
P	193 ± 151^{a}	80.7 ± 81.4^{b}	121 ± 102^{a}
K	74.2 ± 25.6^{a}	63 ± 34.5^{ab}	47.5 ± 19.9^{b}
Essential micro-minerals (mg/kg)			
Fe	$34{,}479 \pm 7{,}755^{a}$	$23{,}997 \pm 9{,}368^{b}$	$30{,}772 \pm 6{,}430^{a}$
Cu	68.4 ± 58.6^{a}	23.3 ± 11.1^{b}	30.2 ± 16.8^{b}
Zn	15.2 ± 12.8^{a}	6.49 ± 7.29^{b}	18.1 ± 12.9^{a}
Ni	0.18 ± 0.05^{b}	0.23 ± 0.03^{c}	0.16 ± 0.04^{a}

Table 7 *(Continued)*

	Phatum Thani	Nakhon Nayok	Chachoengsao
Heavy metals (mg/kg)			
Pb	82.5 ± 31.3[a]	226 ± 135[b]	85.6 ± 11.8[a]
Cd	2.75 ± 0.89[a]	2.11 ± 0.99[a]	1.89 ± 2.73[a]
Hg	5.13 ± 2.61[a]	6.33 ± 1.77[a]	4.86 ± 1.81[a]
Se	1.90 ± 0.12[a]	4.86 ± 11.9[b]	1.20 ± 0.37[c]

Note. [a,b,c] The mean in row differences is significant at the *p-value* < 0.05 level (HSD); N = Nitrogen; P = Phosphorus; K = Potassium; Fe = Iron; Cu = Copper; Zn = Zinc; Ni = Nickel; Pb = Lead; Cd = Cadmium; Hg = Mercury; Se = Selenium

Relationship of Some Elements to Acidic Soil

The results of the correlation of acidic soil with elements Pb, Cu, Cd, Zn, Se, Fe, Hg, Ni, K, P, and N are as follows. In the soil, pH was tested using Pearson's correlation coefficient. Levels of N ($r = -0.606$; $p < 0.01$), Ni ($r = -0.339$; $p < 0.01$), Pb ($r = -0.503$; $p < 0.01$), and Zn ($r = 0.292$; $p < 0.05$) are presented in Table 8. The correlation between N and soil pH is negative because, in soil, N forms ammonium (NH_4^+) and undergoes nitrification by microorganisms present in the hydrogen (H^+) in the environment, which is related to an increase in soil acidity. The correlation of N to soil pH is in accordance with the relationship between soil pH and the C/N ratio ($r = 0.690$; $p < 0.01$), so soil pH is possibly affected by heterotrophic nitrification because a neutral soil pH (pH 6–7) is suitable for microorganism activity (Zhang et al., 2019).

Table 8

Correlation of soil physical properties, mineral soil content, SOC, SOM, and C/N ratio of acidic soil in central Thailand

	pH	BD	Pb	Cu	Cd	Zn	Se	Fe
BD	ns							
Pb	−.503**	ns						
Cu	ns	ns	ns					
Cd	ns	ns	ns	ns				
Zn	.292*	ns	−.255*	.494**	ns			
Se	ns	.247*	ns	ns	ns	ns		
Fe	ns	ns	−.450**	ns	ns	ns	ns	

Table 8 *(Continued)*

	pH	BD	Pb	Cu	Cd	Zn	Se	Fe
Hg	ns	ns	ns	ns	ns	ns	ns	ns
K	ns	ns	ns	ns	ns	ns	ns	ns
Ni	−.339**	ns	.508**	ns	ns	−.330**	ns	−.423**
P	ns	ns	ns	ns	ns	ns	ns	ns
N	−.606**	ns	.573**	ns	ns	−.388**	ns	−.265*
SOM	ns	.273*	ns	ns	ns	ns	ns	ns
SOC	ns	.597**	ns	ns	ns	ns	.254*	ns
C/N ratio	.690**	ns	−.508**	ns	ns	.515**	ns	ns

	Hg	K	Ni	P	N	SOM	SOC	C/N ratio
K	ns							
Ni	ns	ns						
P	−.385**	ns	ns					
N		ns	.547**	ns				
SOM	ns	ns	ns	ns	ns			
SOC	ns	ns	ns	ns	ns	.715**		
C/N ratio	−.251*	ns	−.424**	ns	−.741**	.283*	ns	

Note. *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed); BD = Bulk density; Pb = Lead; Cu = Copper; Cd = Cadmium; Zn = Zinc; Se = Selenium; Fe = Iron; Hg = Mercury; K = Potassium; Ni = Nickel; P = Phosphorus; N = Nitrogen; SOM = Soil organic matter; SOC = Soil organic carbon; C/N ratio = Carbon-to-nitrogen ratio; ns = Not significant

Factors of Acidic Soil Components

Factor analysis of the parameters of the 16 components of acidic soil properties was done by PCA. Prior to this, acidic soil components were tested using Kaiser-Meyer-Olkin (KMO) and Bartlett's test. The KMO Measure of Sampling Adequacy was 0.618 (Table 9), and there was a significant difference in the eigenvalues ($p < 0.001$). The components found in six PCs had an eigenvalue over 1 and explained 71.628% of the total variance in the data set (Table 10). The components had a percentage of the variance of > 10%, as shown in PC1 and PC2. PC1 had 24.047% of the variance (Table 10, Figure 3a). The C/N ratio was the most significant contributor, and factor loading was 0.853, which was selected first. Second were soil pH (0.712) and Zn (0.598), so the correlation between C/N

ratio and soil pH was r = 0.692, and the correlation between C/N ratio and Zn was r = 0.515 (Table 8). PC2 explained 14.887% of the variances (Table 10). SOC was the most significant contributor, and factor loading was 0.873, which was selected first. Second were BD (0.748) and SOM (0.717), so the correlation between SOC and BD was r = 0.597, and the correlation between SOC and SOM was r = 0.715 (Table 8). The relationship of an eigenvalue to components in principle analysis and component loading of PCs is presented in Figure 3(b). Figure 4 shows the cluster analysis for classified groups as a dendrogram. This dendrogram construction presents two major, distinct clusters with four groups from SOC and SOM clustered together in one group and Pb, Se, pH, and C/N ratio in another. The third group comprises Fe, soil moisture, Cu, Zn, percentage of wet material in the soil, and EC. The final group contains P, Hg, and soil temperature. This final group is dissimilar to groups A, B, and C. K and Cd could not be grouped with the others (Figure 4).

Table 9

Results of KMO and Bartlett's test of acidic soil components in central Thailand

Kaiser–Meyer–Olkin Measure of Sampling Adequacy	.618
Bartlett's Test of Sphericity Approximate Chi-Square	367.983
df	120
Sig.	.000

Note. df = Degree of freedom; Sig. = Significant

Table 10

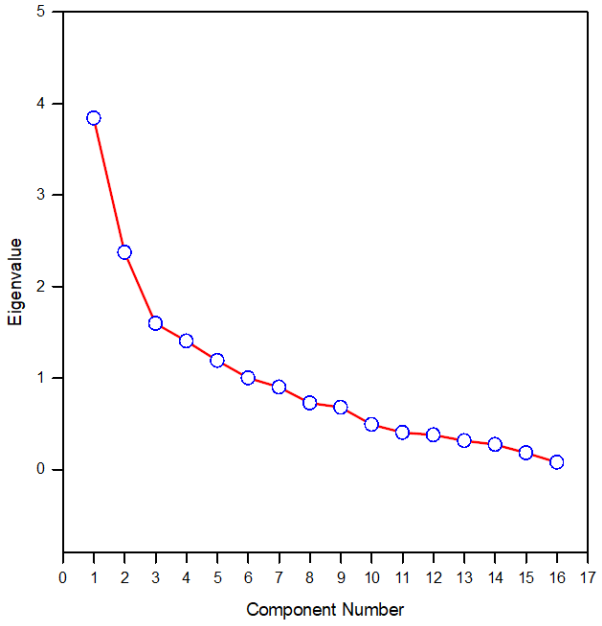
Results of PCA of the statically significance of acidic soil in central Thailand

PCs	Component					
	PC1	PC2	PC3	PC4	PC5	PC6
% of variance	24.04	14.8	10.03	8.83	7.50	6.32
Cumulative %	24.04	38.9	48.9	57.8	65.3	71.6
Eigenvalue	3.84	2.38	1.60	1.41	1.20	1.01
pH	.71	−.08	−.41	−.08	−.02	−.06
SOM	.17	.71	−.36	−.10	−.07	.31
C/N ratio	.85	−.02	−.24	−.17	.08	.09
SOC	.16	.87	−.16	.04	.14	.07

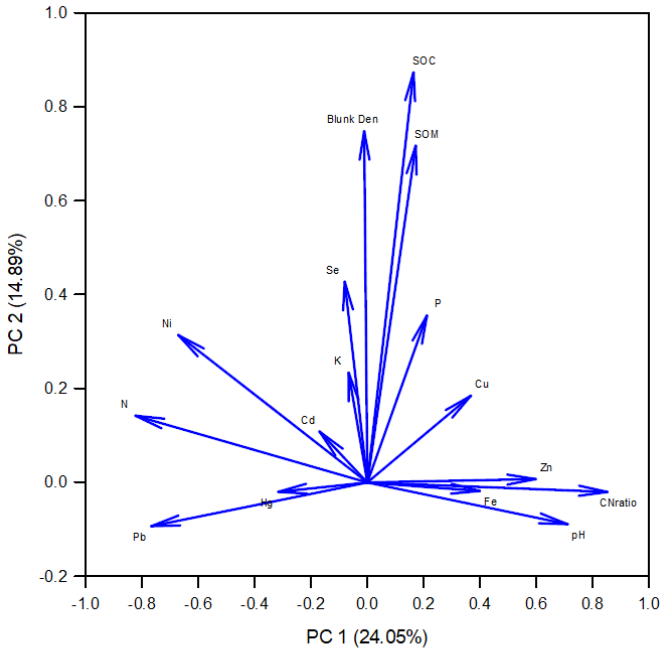
Table 10 *(Continued)*

PCs	Component					
	PC1	PC2	PC3	PC4	PC5	PC6
BD	−.01	.74	.04	.23	.11	−.27
Pb	−.76	−.09	−.00	−.26	.21	.06
Cu	.36	.18	.51	−.03	.47	.19
Cd	−.17	.10	−.02	.56	−.04	.49
Zn	.59	.01	.28	−.31	.48	.09
Se	−.08	.42	−.18	−.12	−.22	−.49
Fe	.39	−.01	.21	.70	−.20	.01
Hg	−.31	−.02	−.22	.45	.64	−.29
Ni	−.67	.31	−.16	−.26	.03	.19
K	−.06	.23	.55	−.01	−.01	−.33
P	.21	.35	.60	−.16	−.36	.07
N	−.82	.14	.18	−.03	.00	.18

Note. PC = Principal analysis; underlined factor loading is weighted higher when within 10% of the variation of the absolute value of the highest factor loading in each PC; SOM = Soil organic matter; C/N ratio = Carbon-to-nitrogen ratio; SOC = Soil organic carbon; BD = Bulk density; Pb = Lead; Cu = Copper; Cd = Cadmium; Zn = Zinc; Se = Selenium; Fe = Iron; Hg = Mercury; Ni = Nickel; K = Potassium; P = Phosphorus; N = Nitrogen



(a)



(b)

Figure 3. Results of PCA for acidic soil components in central Thailand: (a) the eigenvalue of components in principal analysis; (b) the component loading of PCs, so PC1 is carbon-to-nitrogen ratio > pH > zinc (C/N ratio > pH > Zn), and PC2 is soil organic carbon > bulk density > soil organic matter (SOC > BD > SOM)

Physical and Land Use of Acidic Soil in Central Thailand

The soil pH of the study sites in Phatum Thani and Nakhon Nayok is in the extremely acidic group; some Phatum Thani parts have ultra-acidic soil with a pH below 3.5 (Attanandana, 1993; Intorpetch et al., 2014). Nakhon Nayok has a soil pH below 4 (Seeboonruang & Ichikawa, 2007), and Chachoengsao belongs to the strongly acidic group because soil pH in the province is between 4.3 and 6 (Prawach et al., 2017). However, soil pH below 5.5 will affect plant growth (Sumner et al., 1991). Therefore, low pH levels may be an obstacle to agricultural production. Farmers apply manure and water to the soil to prepare the land for planting. Because the study site sits almost entirely inside the irrigation zone, the water supply is directly related to the percentage of wet material in the soil and the soil EC (Lesturgez et al., 2006; Parkpian et al., 1991), supporting the plant growth mechanism.

Influence of soil pH on Some Indicators of Acidic Soil

Soil pH is essential to agricultural production because of its connection to the biogeochemistry of plant and

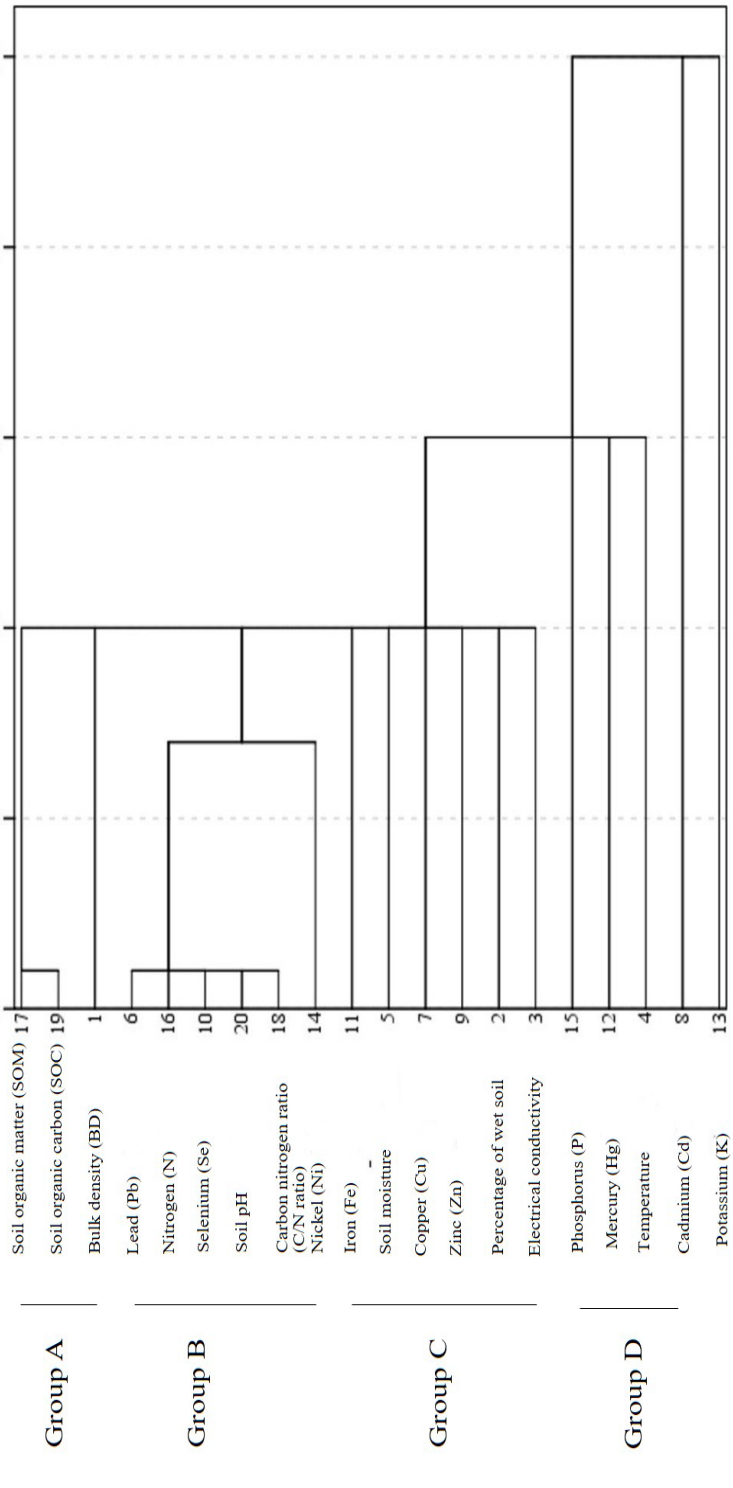


Figure 4. Dendrogram of hierarchy cluster analysis based on acidic soil components of central region parameters

microorganism activity in the soil (Neina, 2019). Components of soil pH, SOM, SOC, and C/N ratio are related to iron in the earth due to ferrous disulfide (FeS_2). In a test to determine the influence of soil pH on components related to C/N ratio > N > Ni > Pb > Zi, it was found that the acidic soil contained large amounts of Fe and Al oxides (Kamprath & Smyth, 2005). Kawaguchi and Kyuma (1974) reported that Fe, Mn, and Si in the central region were higher than in other parts of the country. Results of a similar study showed that the quantity of Fe was higher than that of other elements (Table 3). However, the correlation between the pH of acidic soil and Pb, Zn, and Ni may be due to the parent materials of the soil (Anderson, 1988; B. Liao et al., 2005; Brogowski et al., 2014; Nair & Cottenie, 1971). The soil's parent material in Thailand's central region is non-calcareous sediment, sulfur derived from pyrite, and clay, whose components are kaolinite, muscovite/illite, and smectite (Office of Soil Survey and Land Use Planning, 2010; Spaargaren et al., 1981).

The pH of acidic soil often varies depending on soil level (Arunrat et al., 2017; Kavinchan et al., 2015; Oechaiyaphum et al., 2020; Perie & Ouimet, 2007) and is related to the C/N ratio. Similarly, Tonon et al. (2010) explained that soil pH could influence the transformation of organic matter in the soil. However, SOM and SOC are grouped with BD in the dendrogram (Figure 4). Furthermore, it is connected to the result of factor analysis (Figure 3), meaning that there is a positive relationship between SOM, SOC, and BD ($p < 0.05$).

Factors of Soil Organic Matter and Soil Organic Carbon in Some Components of Acidic Soil

The study shows SOM and SOC in directly related to BD in the dendrogram (Figure 4) of cluster analysis. PCA and component loading of SOM, SOC, and BD are significant components of PC2 (Figure 3). Therefore, generally, SOM must relate to SOC because it uses a percentage of carbon in its calculation (Han et al., 2018; Y. Liao et al., 2015). However, the relationship between SOM, SOC, and BD is closely related to the organic and inorganic materials in the soil content (Perie & Ouimet, 2007) and soil type (Athira et al., 2019; Sakin, 2012). Therefore, SOM is important in understanding the behaviour of microbial activity in soil (Gmach et al., 2019; Grand & Luvkulich, 2012) because it is related to SOC and total nitrogen in the ecology zone. Nevertheless, Sun et al. (2017) reported that capillary water, SOC, and nitrogen have positive interactions with the C/N ratio, and the C/N ratio will decrease with an increase in soil pH. Therefore, the C/N ratio can explain certain minerals in the area (Hamilton et al., 2003; Yang et al., 2019).

Soil Fertility in Acidic Zones of Central Thailand

This study focused on N, P, K, Fe, Cu, Zn, and Ni. The quantity of Fe and Cu is high in acidic soil (von Uexküll, 1986). Gazey and

Davies (2009) explained that pH is inversely related to nutrient availability of Fe, Cu, and Zn. Conversely, N and K increase with pH, and P is optimised at a pH of 6.5. At the study site, however, the number of essential macro and microelements was high compared with the ideal soil for plants (Table 11). Abundant soil is a valuable element for plants. This study analysed and found a high quantity of N, K, Fe, Cu, Zn, and Ni in crude soil samples. The level of P availability in plants was determined using the Bray II method.

Table 11

Soil element quantity compared with ideal (abundant) soil for plants

Elements (mg/kg)	Study site	Shehata and El-Ramady (2012)	Brunetti (1950)	FAO	Mahler (2004)
Fe	29,765	53,600	50–100	50,000[B]	50–10,000
Cu	40.5	60	2–5	70[B]	2–20
Zn	13.3	70	6–12	80[B]	10–100
Ni	0.19	84	-	100[B]	-
N	782	100–200	30,000–50,000	30,000–34,000[A]	10,000–50,000
P	131	30–50	30,000–50,000	1,100–1,200[A]	1,000–5,000
K	61.3	10–200	40,000–50,000	1,800–2,300[A]	5,000–50,000

Note. A represents information in Food and Agriculture Organization of the United Nations (FAO) (1980), and B represents information in FAO (1979); Fe = Iron; Cu = Copper; Zn = Zinc; Ni = Nickel; N = Nitrogen; P = Phosphorus; K = Potassium

Heavy Metal Contamination in Acidic Soil in Central Thailand

The soil collected in the agricultural area was tested for heavy metals Pb, Cd, Hg, and Se. The contamination level did not exceed the standards set by the Pollution Control Department (2004) for soil in habitat and agricultural areas. The level of Pb did not exceed any of the limits shown in Table 12, but Cd and Hg surpassed the limit according to reports by Crommentuijn et al. (1997) and the FAO (2005), and Se exceeded the limit set by Crommentuijn et al. (1997). However, Pb has a negative correlation to soil pH ($r = -.503$; $p < 0.05$), so it may stimulate fungi activity in acidic soil (Lenart & Wolny-Koladka, 2013). B. Liao et al. (2005) discovered that the relationship between a high level of Cd and its correlation with soil pH is related to soil EC.

Table 12

Limits of heavy metal contamination in soil

Elements (mg/kg)	Study site	Thailand[1]	Raymond and Felix (2011)	Crommentuijn et al. (1997)	FAO (2005)
Pb	130	400	600	140	200
Cd	2.25	37	100	1.6	1
Hg	5.43	23	270	2.2	2
Se	1.83	390	ND	0.81	20

Note. [1]Pollution Control Department standard for soil quality in habitat and agricultural areas; Pb = Lead; Cd = Cadmium; Hg = Mercury; Se = Selenium; *ND* = No data

CONCLUSION

The evaluation of soil acidity in central Thailand found an average pH of 4.71 ± 0.87. The soil acidity level can be categorised as very strongly acidic in Phatum Thani and Nakhon Nayok and strongly acidic in Chachoengsao. Soil pH and BD are about 0.34 g/cm^3 in the soil pH relationship to % Wet and EC. The % Wet is related to BD and SOC and SOM value because it is linked to microorganisms in soil surface decomposition activity. However, the dendrogram of hierarchical cluster analysis shows that Se, Ni, and N have similar pH clusters; it also shows that Fe is a majors mineral in soil acidity in central Thailand. The correlation of soil pH to Pb, Ni, N, C/N ratio, and Zn is as follows: PC1 is C/N ratio > pH > Zn, and PC2 is SOC > BD > SOM. Soil pH, SOM, and SOC are similar groups, and soil abundance at the study site contained essential macro and microelements below the ideal level needed for plants. The heavy metal contamination of the acidic soil in the central region did not exceed the standard limit. However, the correlation between SOM and SOC ($r = 0.715$; $p < 0.01$) indicates soil quality and microbial activity.

The acidic soil area in central Thailand is a significant zone for agricultural production in the country. The study found a relationship between the element content and soil physical properties. However, the connection is not strong enough to offer suggestions to farmers who have improved their soil over time because the study found many factors related to soil acidities, such as microorganisms in the area or the chemical behaviour of soil acidity in the country. These factors impact farmers improving the soil by stabilisers such as lime, calcite, and dolomite for adjusting pH in the soil. Therefore, this topic should find practical support in the future, as the search for a solution to soil acidity continues.

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Haplotype Analysis and Phylogeny of *Oryzaephilus surinamensis* Populations from Four Regions in Peninsular Malaysia

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ABSTRACT

The sawtoothed grain beetle, *Oryzaephilus surinamensis*, is a secondary pest that damages rice products and other stored grains. Analysis based on the *cytochrome oxidase subunit I* (*COI*) sequences data, the number of haplotypes (Hap) (*n*), haplotype diversity (*Hd*), haplotype network, genetic distance, and phylogeny between *O. surinamensis* populations from four regions (small-scale), viz. the northern area (Seberang Perai), middle area (Klang), southern area (Pasir Gudang), and east coast (Kuantan) of Peninsular Malaysia, as model sampling locations, were obtained. A total of five haplotypes were detected in all the test populations, two shared (Haplotype 1 and Haplotype 3) and three unique haplotypes (Haplotype 2, Haplotype 4, and Haplotype 5) with haplotype diversity value, *Hd* = 0.6789 were recorded. Furthermore, the neighbour-joining (NJ), maximum parsimony (MP), and Bayesian inference (BI) trees showed a mixture of individuals from all regions in Peninsular Malaysia (Haplotype 1 to Haplotype 4), except Haplotype 5, which was grouped with foreign populations that inherited similar haplotype with those of the European samples. This study assumed a mixture of populations presumably due to human activities and related explicitly to the exportation and importation of rice products across regions. This information is vital for strategising the control management of this pest species to reduce rice storage losses.

Keywords: *COI*, Malaysia, mitochondrial DNA, molecular, phylogenetic analysis, storage pest

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INTRODUCTION

The sawtoothed grain beetle, *Oryzaephilus surinamensis*, is one of the main pests affecting stored grain worldwide (Hashem et al., 2012). This species also has been recorded as the most abundant pest species at a few Klang rice warehouses (Syarifah Zulaikha et al., 2018). In cereal grain commodities, *O. surinamensis* is a secondary pest that infests grains that are already infested by primary pests, such as *Sitophilus* spp. or *Rhyzorpertha dominica* (Trematerra et al., 2012). The adults and larvae of *O. surinamensis* infest various food sources, such as rice, beans, cereals, legumes, flour, and dried fruits (Mowery et al., 2002). Globalisation and international trading activities of commodities have caused the widespread distribution of stored grain insect pests worldwide, especially in the hot climate regions, where the climate is conducive to the growth and development of the pests (Rees, 2004).

Many commercial and synthetic pesticides such as fumigants (Lee et al., 2003) and contact insecticides (Agrafioti & Athanassiou, 2018) have been applied to manage and control the infestation of the stored grain pests. However, long term usage of pesticides would be resulting in residues that will affect the grain quality, human health, and environment (Gahukar & Reddy, 2018). Furthermore, the continuous application of pesticides also induces resistance in this important storage pest, leading to difficulties in its control (Athie & Mills, 2005; Lorini et al., 2007). Thus, a better management process has to be utilised, for example, by using physical (Vincent et al., 2009) and biological (Aman & Yaakop, 2013; Schöller, 2010) methods to manage the storage insect pests below the economic injury level. Some examples of the physical method are by manipulating the moisture content gradient and ventilation to affect the movement of *O. surinamensis*, where the ventilated wet grains would capture a greater proportion of *O. surinamensis* than dry, ventilated grains (Collins & Conyers, 2009). Meanwhile, *Cephalonomia tarsalis* (Hymenoptera: Bethylidae) could suppress the *O. surinamensis* populations (Eliopoulos, 2019) and be an effective biological control method.

In planning an effective management program for this pest species, the population distribution must be understood for strategising protocol. Thus, the molecular approach using haplotype analysis is one of the methods to investigate the status of the pest species distribution (genetic distribution or gene flow) and the relationships among and between populations (Palraju et al., 2018). For example, one of the molecular studies reported on *O. surinamensis* showed no genetic differences in using 16S ribosomal DNA. However, the genetic separation was revealed by using AFLP and two mutations at the *COI* (Sharaf et al., 2013). On the other hand, *Oryzaephilus surinamensis* showed no genetic differences in using 16S ribosomal DNA. Nevertheless, the genetic separation did occur, as revealed by using AFLP and two mutations at the *COI*. Other than that, Govindaraj et al. (2014) showed genetic variations between two populations

of *O. surinamensis* by using RAPD markers due to host characteristics. Based on the positive results of AFLP, RAPD, and COI markers, haplotype analysis using *COI* was performed in this study to investigate the distribution of *O. surinamensis* and relationships among populations sampled from different locations throughout the country.

With the current rising demand for effective pest control of storage food products to ensure food security and safety, it is better to understanding the insects' relationships among populations across regions. Thus, it will contribute significantly to improving the current management strategy for controlling major insect pests. Hence, this study aims to measure the haplotype analysis, illustrate the haplotype network, and construct a phylogeny of *O. surinamensis* populations from Peninsular Malaysia.

METHODS

Sampling Sites

Fresh samples of *Oryzaephilus surinamensis* were collected from four different localities or regions, i.e. Klang (middle area), Kuantan (east coast), Seberang Prai (northern area), and Pasir Gudang (southern area) in Peninsular Malaysia, from June 2016 until July 2017 (Figure 1). This sampling method is considered model sampling for small-scale regions because the distance between sampling sites is less than 1,000 km apart.

Figure 1. Localities of *Oryzaephilus surinamensis* collected from four different regions in Peninsular Malaysia

Insect Sampling

A total of 20 individuals of *O. surinamensis* were successfully collected from four regions, comprising five individuals from each sampling site. According to Goodall-Copestake et al. (2012), a total of five individuals from each population are sufficient for the first assessment of genetic diversity. Active sampling was applied throughout the sampling process. Fresh samples were kept in 100% alcohol to prevent DNA degradation and avoid contamination. The samples were identified until species level using a stereomicroscope (Zeiss Stemi DV4, Germany) and by referring to the key to species by Rees (2004). Further action was taken by keeping the samples in the freezer at -20°C for molecular work. The data on temperature and humidity have been standardised in all the warehouses.

DNA Extraction and PCR Amplification

DNA was extracted from each sample using the NucleoSpin® DNA Insect Kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instruction. The whole body of *O. surinamensis* was used to maximise the DNA volume. Forward and reverse primers chosen were LCO14905'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'(Folmer et al., 1994) to amplify the *cytochrome oxidase subunit I* (*COI*) using a Mastercycler Nexus (Eppendorf, United States of America). The PCR was performed by using a mixture of 12.5 µl of Green Mastermix (Promega, United States of America), 7.5 µl of ddH_2O, 3 µl of template DNA, and 1.5 µl of primer region each from forward and reverse site with the total volume was 25 µl. The PCR process completed each cycle by undergoing a few steps included 3 min of 95°C pre-denaturation, 30 cycles of denaturation for 30 sec at 95°C, 1 min of annealing at 47°C, 30 sec of elongation at 72°C and 10 min of final elongation at 72°C (Halim et al., 2017, 2018). All the PCR products of *O. surinamensis* were sent to Apical Scientific (Malaysia) for sequencing.

Sequences Editing, BLAST, BOLD Analyses, and Sequences Alignment

COI sequences were edited through Sequencher v4.1 software by combining forward and reversed primer and forming a complete sequence. The Barcode of Life Data System (BOLD) (Ratnasingham & Hebert, 2007) and Basic Local Alignment Search Tool (BLAST) (Benson et al., 2013) were used for a comparison between both sample and database sequences based on its evenness percentage and small probability value E (Krauthammer et al., 2000). ClustalW and MEGA7 were applied to align the sequences with the outgroup *Oryzaephilus mercator* (MG458965.1).

Haplotype Analysis and Haplotype Network

Haplotype analysis was performed for the *COI* sequences using DNA Sequence Polymorphism (DnaSP) software, v6 (Rozas et al., 2017). Haplotype diversity (*Hd*) and the number of haplotypes (*n*)

were two parameters analysed for the haplotype analysis. For the minimum-spanning network (MSN), Network 4.6.1.2 was used (Bandelt et al., 1999).

Phylogenetic and Genetic Distance Analyses

For phylogenetic analysis, three sequences from the Genbank database were selected, which were KM446924.1 (Germany), KU4942022.2 (France), and KC129180.1 (Finland) to aid the analysis. The neighbour-joining tree was constructed using PAUP* v4.0b10 for 1,000 replications using the Kimura two-parameter (K2P) model. Maximum parsimony (MP) tree was performed using PAUP* v4.0b10 with 100 stepwise additions. Bayesian inference was constructed using MrBayes 3.2 (Ronquist et al., 2012). jmodelTest 3.7 (Darriba et al., 2012) was run to get the Akaike's Information Criterion (AIC) calculation to select the best model to construct the Bayesian tree. The analysis was run using 1 million generations, and Bayesian tree topology is considered entirely constructed when the posterior probability was <0.01. The phylogenetic trees were visualised in Tree view 3.0, with *Oryzaephilus mercator* (MG458965.1) as an outgroup. Genetic distances within and between populations were performed using Kimura 2-Parameter (K2P) in MEGA7 software.

RESULTS

Sequence Variation

A total of 20 samples of *O. surinamensis* were successfully extracted in this study, and 620 bp of the *COI* was successfully amplified from each individual. Each sample was recorded in the Genbank, and their accession numbers were listed in Table 1 according to their locations.

Table 1

Information on accession number of Oryzaephilus surinamensis *from the Genbank*

Species	Code	Accession number	Sampling site
Oryzaephilus surinamensis	JASAKlang1	MH587653	Klang, Selangor
Oryzaephilus surinamensis	JASAKlang2	MH587654	Klang, Selangor
Oryzaephilus surinamensis	JASAKlang3	MH587655	Klang, Selangor
Oryzaephilus surinamensis	JASAKlang4	MH587656	Klang, Selangor
Oryzaephilus surinamensis	JASAKlang5	MH587657	Klang, Selangor
Oryzaephilus surinamensis	JYAJohor1	MH587658	Pasir Gudang, Johor Bahru

Table 1 *(Continued)*

Species	Code	Accession number	Sampling site
Oryzaephilus surinamensis	JYAJohor2	MH587659	Pasir Gudang, Johor Bahru
Oryzaephilus surinamensis	JYAJohor3	MH587660	Pasir Gudang, Johor Bahru
Oryzaephilus surinamensis	JYAJohor4	MH587661	Pasir Gudang, Johor Bahru
Oryzaephilus surinamensis	JYAJohor5	MH587662	Pasir Gudang, Johor Bahru
Oryzaephilus surinamensis	MISCPenang1	MH587663	Seberang Prai, Pulau Pinang
Oryzaephilus surinamensis	MISCPenang2	MH587664	Seberang Prai, Pulau Pinang
Oryzaephilus surinamensis	MISCPenang3	MH587665	Seberang Prai, Pulau Pinang
Oryzaephilus surinamensis	MISCPenang4	MH587666	Seberang Prai, Pulau Pinang
Oryzaephilus surinamensis	MISCPenang5	MH587667	Seberang Prai, Pulau Pinang
Oryzaephilus surinamensis	EMAKuantan1	MH587668	Kuantan, Pahang
Oryzaephilus surinamensis	EMAKuantan2	MH587669	Kuantan, Pahang
Oryzaephilus surinamensis	EMAKuantan3	MH587670	Kuantan, Pahang
Oryzaephilus surinamensis	EMAKuantan4	MH587671	Kuantan, Pahang
Oryzaephilus surinamensis	EMAKuantan5	MH587672	Kuantan, Pahang

Five haplotypes with 11 bp were defined from four populations of 20 individuals of *O. surinamensis* (excluding outgroup). Only the Kuantan population had three haplotypes (Hap 1, Hap 3, and Hap 5) in which Hap 5 was the unique haplotype. At the same time, Hap 1 and Hap 3 were shared between the Klang and Penang populations. In addition, both Klang and Johor samples have unique haplotypes (Hap 2 and Hap 4). The haplotype diversity (*Hd*) was 0.6789 (Table 2).

In Figure 2, Hap 5, represented by two individuals from Kuantan, showed the most extended mutational steps compared to the other haplotypes. Meanwhile, Hap 1 showed the largest circle, a feature shared by three populations, i.e. Klang, Seberang Prai, and Kuantan, continued by Hap 3 as recorded from the sample populations collected from

Table 2

Segregating sites (11bp) in 620 bp of COI *marker defining five haplotypes of* Oryzaephilus surinamensis *populations*

Haplotypes	Nucleotides positions				Locality			
					1 Klang	2 Pasir Gudang	3 Seberang Prai	4 Kuantan
		1 2 2	2 3 4	5 5				
		6 0 0	5 0 4	1 4				
	6 8 9	8 1 4	8 4 1	7 0				
Hap_1	T G A	A T G	C A A	A A	4		4	2
Hap_2	G A G	. . .	. . .	. .	1			
Hap_3	. . .	G . .	. . .	. .		4	1	1
Hap_4	. . .	G . .	. G .	. .		1		
Hap_5	. . .	. C A	T G T	G G				2

Note. A = Adenine; C = Cytosine; G = Guanine; T = Thymine

Pasir Gudang, Seberang Prai, and Kuantan. On the other hand, both Hap 2 and Hap 4 have the smallest circle because each was represented by one individual only.

Genetic Distance and Phylogenetic Trees

The highest genetic distance was showed between Hap 2 - Hap 5 with 91%. Meanwhile, the lowest genetic distance was between Hap 1 - Hap 3 and Hap 3 - Hap 4 with 9%. Among all the haplotypes, Hap 5 showed a higher genetic distance with all the other haplotypes, 64%, 91%, 73%, and 64%. In comparison, the combination of other haplotypes ranged between 9% - 45% (Table 3). From Figure 3, Pasir Gudang–Seberang Prai showed the nearest clade with the lowest genetic distance is 0.001. Meanwhile, Pasir Gudang–Kuantan and Klang–Kuantan showed that the farthest clade with genetic distance is 0.006 (Table 4).

Neighbour-joining (NJ) tree constructed showed a bootstrap value ranging between 50% - 100%. However, all individuals from each population were not successfully

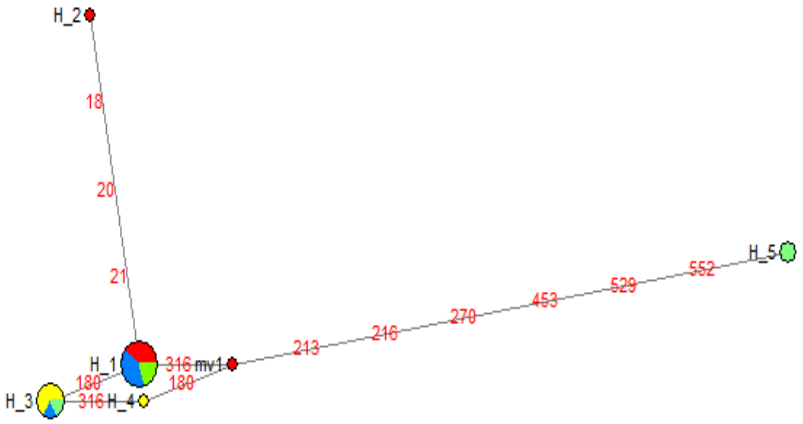


Figure 2. Haplotype network based on *COI* dataset

Table 3

Percentage of sequence differences between pairs of haplotypes

Haplotype	Distance (%)
Hap 1 - Hap 2	27
Hap 1 - Hap 3	9
Hap 1 - Hap 4	18
Hap 1 - Hap 5	64
Hap 2 - Hap 3	36
Hap 2 - Hap 4	45
Hap 2 - Hap 5	91
Hap 3 - Hap 4	9
Hap 3 - Hap 5	73
Hap 4 - Hap 5	64

grouped into a similar group or population. While two individuals, EMA 1 and EMA 5 from Kuantan, form a paraphyletic clade with other EMA individuals by 100% bootstrap value (Figure 3). Both EMA 1 and EMA 5 had seven similar nucleotides, which caused them to be grouped and separated from others. The MP supported the defined separation of the Kuantan populations with 98% bootstrap value. Bayesian inference tree was constructed with a model of GTR+G through jmodelTest 3.7. The

topology showed EMA 1 and EMA 5 grouped with Germany, France, and Finland samples, which they located at the basal of the tree with posterior probability (pp) 0.98. These two individuals had separated with a new clade with pp = 0.89 and formed a subclade with pp = 0.80 (Figure 4).

Table 4
Genetic distance of Oryzaephilus surinamensis *between populations*

Location	Distance
Klang - Seberang Prai	0.002
Klang - Kuantan	0.006
Klang - Pasir Gudang	0.003
Pasir Gudang – Seberang Prai	0.001
Pasir Gudang - Kuantan	0.006
Seberang Prai - Kuantan	0.005

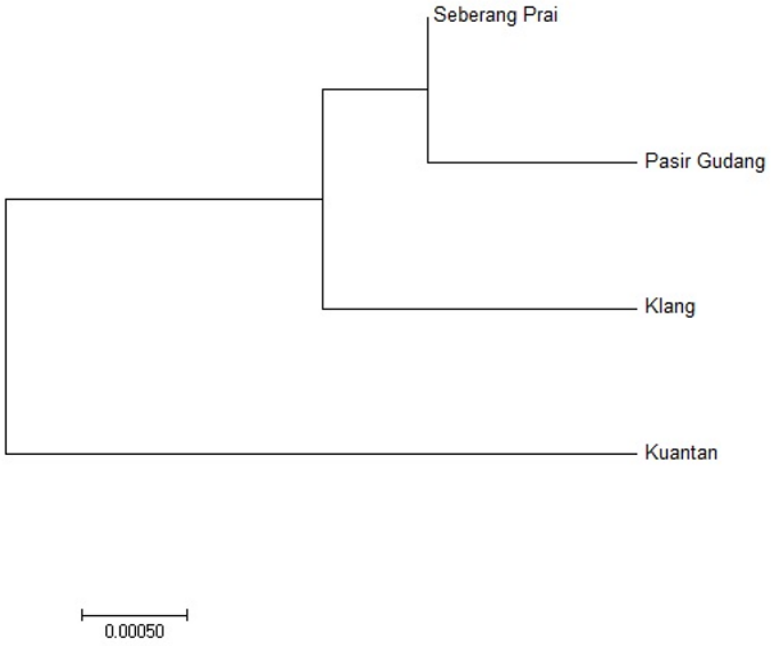
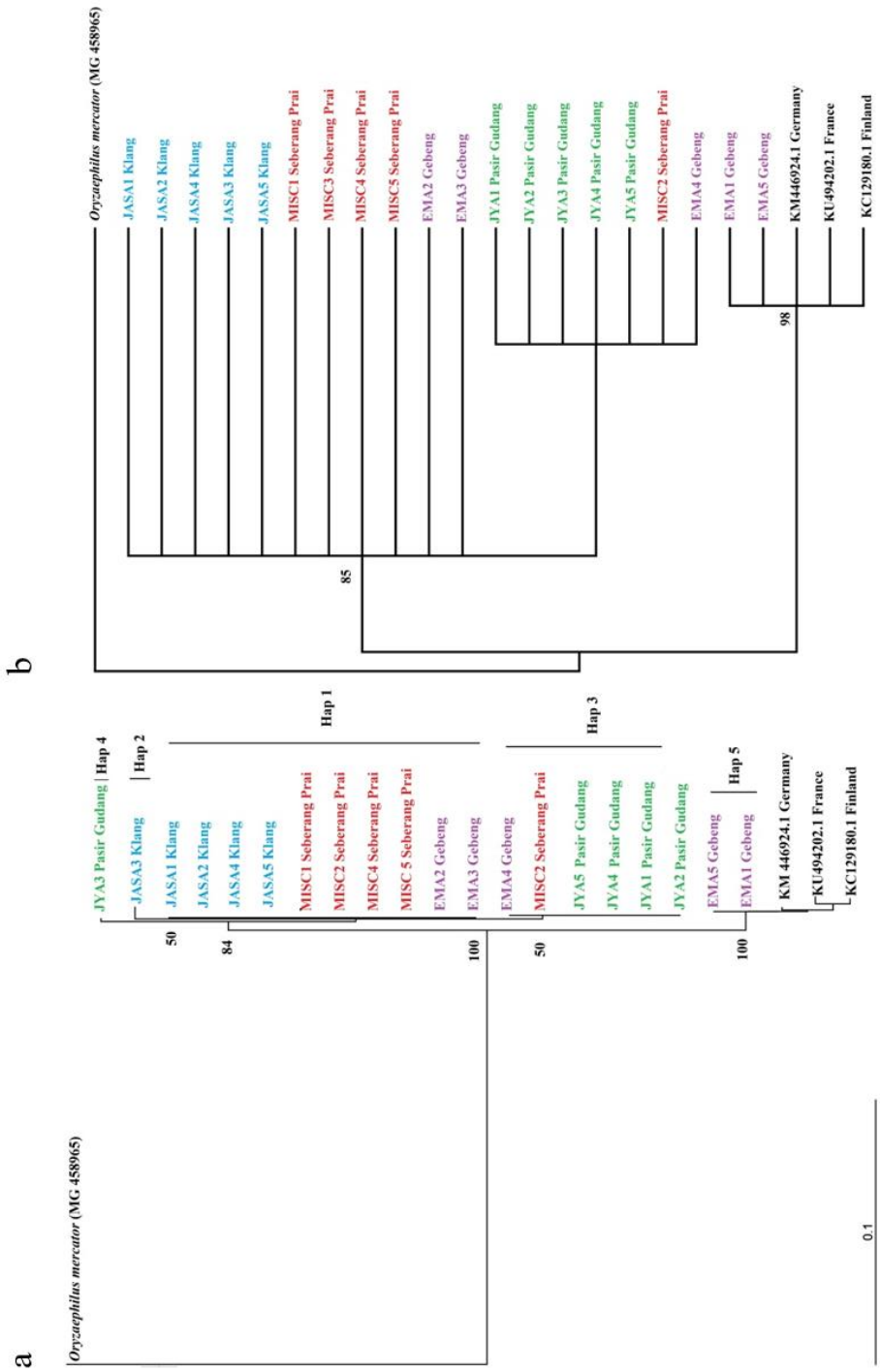


Figure 3. Population tree by haplotype sequence among four populations


a
Oryzaephilus mercator (MG 458965)
JYA3 Pasir Gudang
JASA3 Klang
JASA1 Klang
JASA2 Klang
JASA4 Klang
JASA5 Klang
MISC1 Seberang Prai
MISC2 Seberang Prai
MISC4 Seberang Prai
MISC 5 Seberang Prai
EMA2 Gebeng
EMA3 Gebeng
EMA4 Gebeng
MISC2 Seberang Prai
JYA5 Pasir Gudang
JYA4 Pasir Gudang
JYA1 Pasir Gudang
JYA2 Pasir Gudang
EMA5 Gebeng
EMA1 Gebeng
KM 446924.1 Germany
KU494202.1 France
KC129180.1 Finland
Hap 4
Hap 2
Hap 1
Hap 3
Hap 5
50
84
100
50
100
0.1
b
Oryzaephilus mercator (MG 458965)
JASA1 Klang
JASA2 Klang
JASA4 Klang
JASA3 Klang
JASA5 Klang
MISC1 Seberang Prai
MISC3 Seberang Prai
MISC4 Seberang Prai
MISC5 Seberang Prai
EMA2 Gebeng
EMA3 Gebeng
JYA1 Pasir Gudang
JYA2 Pasir Gudang
JYA3 Pasir Gudang
JYA4 Pasir Gudang
JYA5 Pasir Gudang
MISC2 Seberang Prai
EMA4 Gebeng
EMA1 Gebeng
EMA5 Gebeng
KM446924.1 Germany
KU494202.1 France
KC129180.1 Finland
85
98

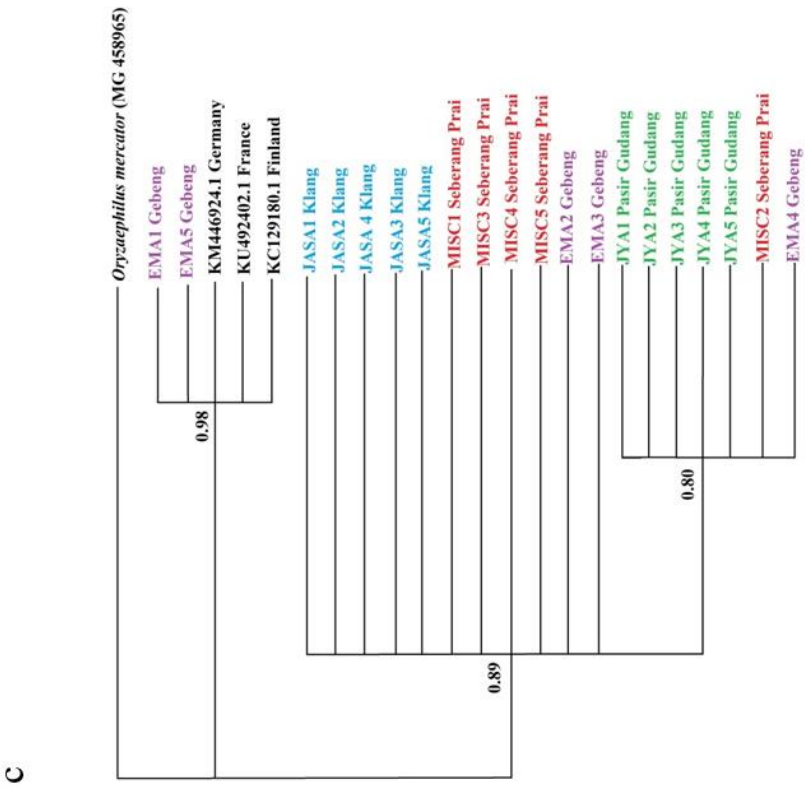


Figure 4. (a) The bootstrap tree resulted from neighbour-joining (NJ) analysis of *Oryzaephilus surinamensis COI* sequences data based on 1,000 replications; (b) The bootstrap tree resulted from maximum parsimony (MP) analysis of *O. surinamensis COI* sequences data based on 1,000 replications. Values above the lineages are the bootstrap values; (c) The Bayesian tree resulted from Bayesian inference (BI) analysis of *O. surinamensis COI* sequences data. Values at the internal nodes are the posterior probabilities (pp)

DISCUSSION

The mtDNA has been widely used to detect the genetic diversity, genetic differentiation, and population structure of organisms, e.g. study by Ambrose et al. (2012), Annan et al. (2007), Dyer et al. (2008), and Wellenreuther et al. (2011). The mtDNA *COI* marker efficiently measures the genetic distance of test populations either within or between the populations. Notably, several studies have suggested that *COI* is an ideal marker for examining interspecific or intraspecific phylogenetic relationships among insects (Ahmed et al., 2009; Sole et al., 2008). Furthermore, this marker has also been used widely as the barcoding marker in identifying and discriminating storage pest species for Integrated Pest Management (IPM) purposes (Ghazali et al., 2014; Halim et al., 2017, 2018). Therefore, despite the small number of samples implemented in the genetic diversity analyses, the data were sufficient for genetic diversity analysis. Furthermore, a study by Amzati et al. (2018) and Gollner et al. (2016) also showed that several samples implemented for the analysis did not significantly affect the study results.

The geographical factors, such as landscape, latitude, longitude, and environmental factors (temperature and precipitation), can affect a species' genetic and population structures (Pauls et al., 2013; Wellenreuther et al., 2011). The populations located at the western coast of Peninsular Malaysia were shypothesised to differ distinctly from those of the eastern coast because of the physical barrier created by the Main Range (Banjaran Titiwangsa) as in many animal species. So, the exact condition will happen for the *O. surinamensis* from four geographical ranges at the west and east-coast of Peninsular Malaysia. Several studies on weak-flying insects support this information. For example, Cheng et al. (2014) reported on various termite species populations, *Coptotermes gestroi,* that exhibited haplotypes differences between the west and east coasts of Peninsular Malaysia (area sites) using *COI* and 16S sequences.

A study by Sum et al. (2014) also recorded the distinct haplotype separation between the northern and central populations of Anopheles sinensis that were geographically isolated by the mountain ranges of Gunung Tahan and Banjaran Titiwangsa (Sankalakhiri Range). Notably, most beetle species are generally weak flyers (Tuda et al., 2014). The adults of *O. surinamensis* could hardly fly anywhere far (Heaps, 2006). Thus, this study predicts that all individuals from the same population are grouped and have similar haplotype number. However, our finding contradicted the hypothesis, in which all individuals were found to be pretty well mixed up between populations. It is strongly indicated that some other factors besides the physical barrier had likely contributed to this situation. Based on this finding, that situation is not related to insect storage pests. In contrast, the distribution of the species is probably affected by the human or commodity movement (S. Yaakop, personal communication, July 10, 2017).

The results showed that the mixture of populations had likely occurred between the four locations for most of the study samples (Figure 4). The results have been supported by NJ, MP, and BI trees, which showed that all populations from west-central, southern, northern, and eastern areas were mixed. This observation was also supported by the high to low bootstrap values and posterior probabilities. The maximum distance between two locations is about 630 km, for example, on Seberang Prai-Pasir Gudang. This result was supported by Mazzi and Dorn (2012) in their study of the herbivorous insect pests for contemporary agro ecosystems and by Wei et al. (2015) for the oriental fruit moth, *Grapholita molesta* infesting on stone and pome fruits, with observations that genetic exchange may happen due to human-mediated dispersals and transport by the host plants over a huge area of geographic distance.

At all the warehouses in this study, there were active rice exportation and importation activities from one warehouse to another within the region. Likewise, human activities, migration, and gene flow were the main factors affecting the genetic study and population structure besides climate change and other environmental and ecological factors. The *O. surinamensis* also a widespread pest due to the infestation that involves manufacturing, storage, and retail phases (Nurul-Huda & Noor-Amni, 2020). Here, human activities were concluded as one of the main reasons for the population mixing of the pest species. The haplotype analysis and haplotype network (Table 2; Figure 2) showed that the Klang, Seberang Prai, and Kuantan populations shared similar haplotypes for specific individuals.

In our study, this haplotype appeared in four individuals from Klang, four from Seberang Prai, and two from Kuantan. Furthermore, Hap 3 was also shared between three locations, i.e. Pasir Gudang, Seberang Prai, and Kuantan. Thus, the genetic exchange had likely occurred between the populations of the eastern (Kuantan) and western parts of Peninsular Malaysia (Seberang Prai, Klang, and Pasir Gudang) (Table 3). Hap 5 indicated that it was split from the old population with one ancestral haplotype and carried the alleles that were inherited together with the foreign samples (European populations) caused by mutations (Doorenweerd et al., 2020).

In NJ, MP, and BI topology, these groups formed a polytomy showing unresolved relationships between them (Figure 4). Interestingly, two samples from Kuantan presented one unique haplotype, which was formed and separated from the main clade of all the samples from all populations. It is suggested that the information of two individuals from Kuantan was unique and distinct from other samples or haplotypes (Table 2). Clustering of two individuals EMA1 and EMA5, with the samples from GenBank could indicate that these alleles were inherited together with the samples from Finland, Germany, and France (European). It showed that *O. surinamensis* was not native from Malaysia and was described by Linnaeus in 1786 in Suriname, in the northern part of South America (Champ & Dyte, 1976). However, it might have originated from ancient Greece based on the paleontological record in 7000 BC

due to the presence of stored product stock such as wheat, etc. (Llobera, 2002).

Generally, different varieties of rice stocks from several countries worldwide were imported and delivered to the Malaysian retailers through several harbours in Peninsular Malaysia. From there, the stock would be sorted out and divided into several warehouses in the country. In this regard, it is very imperative to obtain accurate information on to the origin of the insect populations in international trade to effectively manage and control the pest infestations related to our food security and safety. Therefore, it is also supported by Fleurat-Lessard and Pronier (2006) to apply genetic data through random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique to understand the population level of pest species towards international trade.

CONCLUSION

This study has successfully presented the phylogeny, haplotype data, and haplotype network of *Oryzaephilus surinamensis* populations based on *COI* sequences from four localities or regions in Peninsular Malaysia. Five haplotypes were obtained, of with Hap 5 showing uniqueness, inherited together or shared with the foreign samples (European populations). The mixture of the populations revealed a close relationship of this species across the east and west coast populations. It was most probably due to human activities by rice exportation and importation from one warehouse to another within the regions. This study could provide the initial and fundamental data with the potential to be used in IPM sto strategise the control management of important pest species. Additional molecular markers and more individual samples per locality are suggested to be added for more comprehensive data. Implementing the population genetic study is also highly necessary to draw a solid conclusion soon.

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Small Pteropodid Bats are Important Pollinators of Durian in Terengganu, Malaysia

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ABSTRACT

Bats are often misunderstood as agricultural pests and have received little attention for conservation efforts. However, bats are critical pollinators to commercially important agricultural products, such as durians. This study intends to confirm the role of small pteropodid bats as pollinating agents to flowering durian trees. Samplings were conducted in April 2018 to record bats visiting the flowers of two durian species, *Durio zibethinus* and *Durio lowianus* at Malaysian Agricultural Research and Development Institute (MARDI) Jerangau, Terengganu. Captured bats were swabbed for conspecific pollen load on their bodies to determine their potential role as pollinators. One hundred thirty-one (131) pollen swabs were collected from three pteropodid bat species: *Eonycteris spelaea* Dobson, *Cynopterus brachyotis* Dobson, and *Cynopterus horsfieldii* Gray. Only *E. spelaea* and *C. brachyotis,* however, were found with conspecific pollen loads on their bodies. Between the two, *E. spelaea* showed a higher potential to be the pollinating agent for the durian trees. Hence, they recorded more individuals carrying many conspecific pollen grains while visiting the trees. Thus,

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this finding will hopefully reduce the misconceptions held on bats and conserve them in Malaysia.

Keywords: Conspecific pollen, *Cynopterus brachyotis*, *Durio lowianus*, *Durio zibethinus*, *Eonycteris spelaea*, pollen load, pollinating agent

INTRODUCTION

From 18 bat families worldwide, only two families: the Pteropodidae, which occurs in Paleotropical regions and the Phyllostomidae, which occurs in the Neotropical regions, are specialised floral visitors (Fleming et al., 2009). These bats have been identified as very active and regular flower visitors of many plant species, transporting large pollen loads on different parts of their bodies (Heithaus et al., 1974; Sazima & Sazima, 1978). Consequently, they are critical pollinating agents for approximately 250 plant genera (Fleming et al., 2009).

Many of the plants pollinated by bats (known as chiropterophilous plants) are endemics (Fleming & Muchhala, 2008), and many are of considerable economic value (Bumrungsri et al., 2013; Fujita & Tuttle, 1991; Kunz et al., 2011). In Malaysia and Thailand, for example, the pteropodid bats were reported as pollinating agents for ecologically and economically important plants such as durian (*Durio zibethinus*), bananas (*Musa* spp.), Indian trumpet (*Oroxylum indicum*), kapok tree (*Ceiba pentandra*), bitter bean (*Parkia speciosa*), and mangrove apples (*Sonneratia* spp.) (Acharya et al., 2015; Bumrungsri et al., 2013; Lim et al., 2018; Nor Zalipah et al., 2016; Nuevo-Diego et al., 2019; Stewart & Dudash, 2017). Of these, durian was a vital cash crop for both countries. In 2015, for example, Malaysia and Thailand were reported to have exported a total of USD 405 million worth of durian (Mokhzani, 2017).

Despite being economically important pollinating agents, fruit growers deter the pteropodid bats from visiting their fruit crop trees (Aziz et al., 2016). Thus, resulting in failed fruit sets particularly, for the self-incompatible tree species such as durian (Bumrungsri et al., 2009; Lim & Luders, 1998). Therefore, this study highlighted the critical role of the pteropodid bats as pollinating agents in the agricultural area, not only to the durian (Genus *Durio*) trees they visited, but also to the other fruit crops they might visit during their foraging activities. The information gained from this study will help to reduce the misconception of bats as agricultural pests and consequently contribute to the conservation of the bat population in Malaysia.

MATERIALS AND METHODS

Study Site

The study was conducted at the Malaysian Agricultural Research and Development Institute (MARDI) Jerangau (4° 48' 370" N 103° 8' 680" E), located at Hulu Terengganu District in Terengganu, Peninsular Malaysia (Figure 1). The establishment of this station was to conduct research and development related to agriculture, food, and agro-based industries. Apart from office buildings, laboratories, and staff quarters, this station also consists of approximately

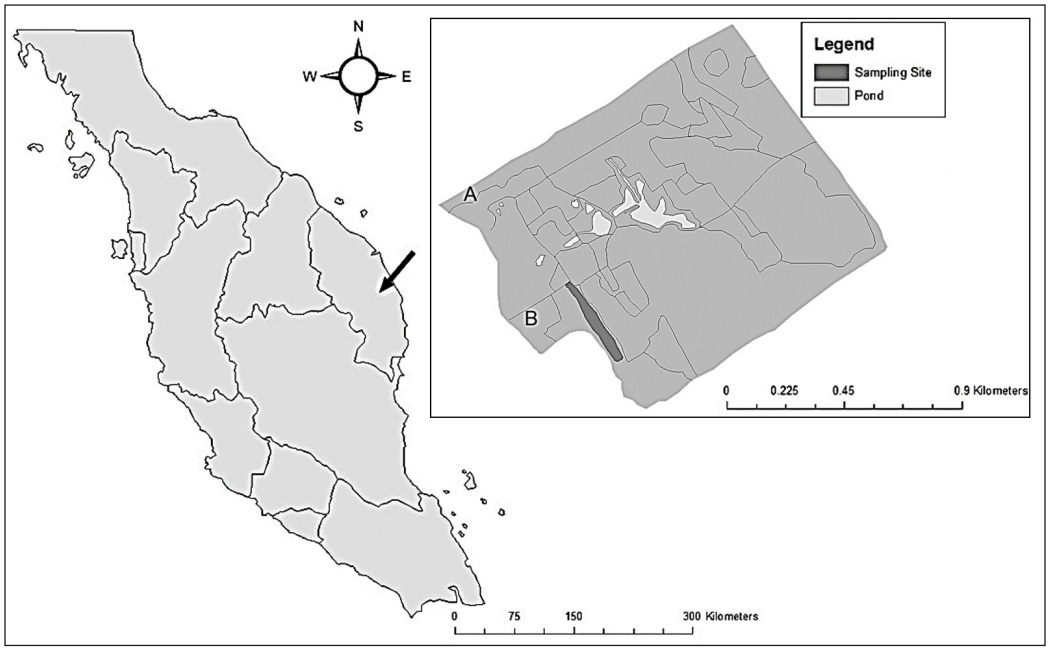

Figure 1. Arrow indicates the location of the sampling site at Hulu Terengganu, Peninsular Malaysia. Inset map shows the durian plantation area near the office (A) and staff quarter's compound; (B) where the mist nettings were conducted

145 ha of an orchard planted with various fruit crops such as durian (*Durio* spp.), banana (*Musa* spp.), mangosteen (*Garcina mangostana*), papaya (*Carica papaya*), and rambutan (*Nephelium lappaceum*) for various research and development activities. Of this area, approximately 145 ha is planted with eight durian species (*Durio dulcis*, *Durio graveolens*, *Durio kutejensis*, *Durio lowianus*, *Durio oxyleyanus*, *Durio oblongus*, *Durio singaporensis*, and *Durio zibethinus*), with the majority being *D. zibethinus* trees, the most cultivated species in the genus of *Durio* (Idris, 2011). At the study area, durian trees of all species were observed to flower simultaneously between March and April every year (N. H. Mohd Zainal Abidin, personal communication, July 22, 2019). This station is surrounded by oil palm plantations manage by Terengganu Development and Management (TDM) Sdn. Bhd. and Federal Land Development Authority (FELDA).

Bat Trapping and Pollen Swab

Bat trappings were conducted on nine nights throughout April 2018, during the flowering time of the durian trees at the study area. We observed the peak flowering of *D. zibethinus* trees for the first three weeks of the month, followed by the peak flowering of *D. lowianus* trees on the fourth week when most of the *D. zibethinus* trees ceased flowering. Thus, trappings were conducted for six nights between April 5th and 21st for bats visiting the flowering *D. zibethinus* trees, and four nights between April 20th and 28th for bats visiting the flowering *D.*

lowianus trees. For each trapping night, a total of 5-14 nylon mist nets (height: 4 m, width: 9 m) were set up at 1800 h with the aid of two aluminium poles and placed close to the flowering trees. The nets were left open throughout the netting nights and were tended to usually in two-hour intervals, starting from 1900 h to 0100 h, and between 0600 h and 0700 h the following day. In Indonesia, the peak visitation of pteropodid bats to durian (*D. zibethinus*) flowers was observed between 2000 h and 2400 h (Sheherazade et al., 2019). Thus, the nets were checked as regularly as possible during this period to avoid distress and potential harm to the netted bats. In total, bat trappings were conducted for 696 and 348 net-hour for *D. zibethinus* and *D. lowianus*, respectively.

When bats were netted, nets were lowered, and the bats were screened for pollen loads. Sheherazade et al. (2019) reported that the head of pteropopid bats was usually in contact with the stigma and anthers while foraging at the durian (*D. zibethinus*) flowers. Therefore, the pollen grains adhering to the bat's head were collected by carefully rubbing cotton wool buds to their heads individually. The cotton wool buds were then kept in a 1 ml centrifuge tube containing 75% ethanol to preserve the pollen grains for pollen identification. Bats were then removed from the net, measured using a plastic vernier calliper (forearm length, ear length, tail length, and tibia length) and weighed using a digital balance (FEJ 600A, Colonial Weighing Australia Pty. Ltd, Australia). Species identification was made following the keys provided by Kingston et al. (2006) and Francis (2008). Before being released at their point of capture, the bats were marked with non-toxic nail polish at the nail of their hind leg to give unique numbering for individual recognition upon recapture (Zulfemi et al., in press). Pollen swabs from recaptured individuals were considered distinct samples.

Pollen Observation and Identification

Pollen grains collected were observed under an optical light microscope (CH20, Metric Optics Sdn. Bhd., Malaysia) in the laboratory. For each sample, 1 µl of ethanol with pollen grains was transferred onto a glass slide using a micropipette for the microscopic observation. The microscope was attached with an eyepiece camera (84 mm length x 23 mm diameter, Dino-eye AM 423X, AnMo Electronics Corporation, Taiwan) to identify pollen. The pollen counts were conducted for ten slides for each sample, and the total number of pollen grains carried by the bats was extrapolated for 1 ml of ethanol. Pollen was identified by comparisons with known pollen types collected at the study area for references and by referring to Mohamed (2014).

Data Analysis

IBM SPSS Statistics (version 20) was used to analyse the data. A comparison of the number of pollen types carried by each bat species was conducted using the Kruskal-Wallis test. In contrast, Friedman's analysis of variance (ANOVA) for repeated measures was used to test the significant difference

(Field, 2013) in the number of pollen grains according to pollen types for *E. spelaea* and *C. brachyotis*. Wilcoxon sign-ranked test was used to determine the significant difference in conspecific and heterospecific pollen loads of *E. spelaea* and *C. brachyotis*. As the durian pollen grains could not be differentiated into their species level from observation under the light microscope, all of them were classified as conspecific. In contrast, the non-viable *Durio* pollen grains were grouped with the other non-durian pollen grains as heterospecific. On the basis that non-viable pollen also does not result in fertilisation (their relatively smaller size distinguished the non-viable *Durio* pollen grains by in comparison to the viable grains, and their translucent appearance when observed under the light microscope). Multiple comparisons (step-down method) were conducted following significant results of Kruskal-Wallis and Friedman's ANOVA tests for the data that violated the normality assumption.

RESULTS AND DISCUSSION

Bat Species Visiting Durian Trees

A total of 118 individuals of pteropodid bats were captured visiting durian trees, consisting of only three species: *Eonycteris spelaea* (lesser dawn bat), *Cynopterus brachyotis* (lesser short-nosed fruit bat), and *C. horsfieldii* (Horsfield's fruit bat). From the total individuals recorded, seven *E. spelaea* individuals and four *C. brachyotis* individuals were recaptured once, with one of *E. spelaea* recaptured twice. Thus, this study recorded 131 captures. The most frequently captured was *E. spelaea* ($\chi^2 = 67.99$, df = 2, $p < 0.05$), 81 captures, followed by *C. brachyotis* with 48 captures. At the same time, the least caught was *C. horsfieldii,* with only two individuals netted (Figure 2). The capture rates calculated was higher for *D. lowianus* (0.24 individuals per net-hour) than for *D. zibethinus* (0.07 individuals per net-hour), with *E. spelaea* as the most commonly caught bat species for both durian species (Table 1).

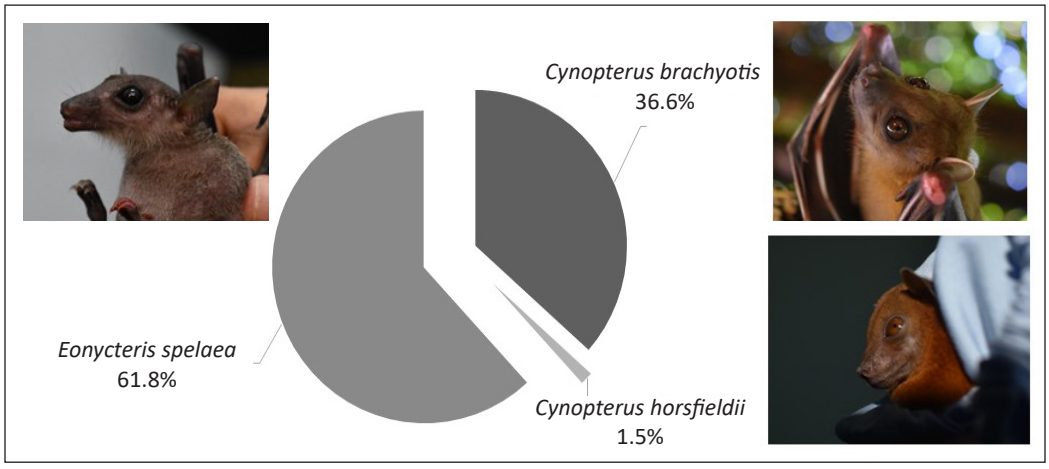

Figure 2. Number of captures (in %) for each bat species recorded at the Malaysian Agricultural Research and Development Institute (MARDI) Jerangau

Table 1
Capture rates (individuals per net-hour) of the pteropodid bats caught visiting durian trees at the Malaysian Agricultural Research and Development Institute (MARDI) Jerangau

Bat species	*Durio zibethinus*	*Durio lowianus*
Eonycteris spelaea (N = 81)	0.0330 (n = 23)	0.1667 (n = 58)
Cynopterus brachyotis (N = 48)	0.0316 (n = 22)	0.0747 (n = 26)
Cynopterus horsfieldii (N = 2)	0.0014 (n = 1)	0.0029 (n = 1)
Total	0.0661 (n = 46)	0.2443 (n = 85)

Note. N represents the number of captures for each bat species, and n represents the number of captures according to bat species for each durian species

Diet could explain the sequence of bat capture frequencies recorded in this study. As the nets were set up near the flowering durian trees, *E. spelaea* individuals were the most captured because it is one of the three specialised nectar-feeding bat species reported in Peninsular Malaysia (Gould, 1978). This bat species are known to feed mainly on floral resources such as nectar, pollen grains and flower petals of the chiropterophilous plants such as durian (Bumrungsri et al., 2013; Lim et al., 2018; Start & Marshall, 1976; Thavry et al., 2017). The other two specialised nectar-feeding bats in Peninsular Malaysia are *Macroglossus minimus* (long-tongued nectar bat) and *Macroglossus sobrinus* (long-tongued fruit bat). Both of which, however, were not recorded visiting the durian flowers in this study.

In contrast to *M. sobrinus*, which is reported as a more inland species, *M. minimus* lives in the coastal areas and has never been recorded away from mangrove areas. *Macroglossus* species are known to roost close to their food resources (within a 2 km radius) and do not commute long distances to feed (Start & Marshall, 1976). Stewart et al. (2014) reported *M. sobrinus* as a flower visitor of durian trees in southern Thailand. Nevertheless, based on our nettings and their small travelling distance from their roosting site, both *Macroglossus* species are suggested, probably absent at our study site. *Eonycteris spelaea*, on the other hand, is a cave dweller known to travel long distances during the night in search of food (Ahmad Yazid et al., 2019; Start & Marshall, 1976), while *C. brachyotis* roosts under large leaves of trees, especially palms (Francis, 2008; Kingston et al., 2006; Tan et al., 1997). Both *C. brachyotis* and *C. horsfieldii* are common and abundant in all habitats, including orchards and plantations (Kingston et al., 2006). The presence of various fruiting and flowering trees at the study site, surrounded by oil palm plantations, provides abundant food resources and offers suitable habitat for these pteropodid bats.

Other than *C. brachyotis* and *C. horsfieldii*, *Cynopterus sphinx* (greater short-nosed fruit bats) and *Rousettus amplexicaudatus* (Geoffroy's rousette) were among the most common pteropodid bats caught visiting flowering durian trees and other chiropterophilous plants such as

banana (*Musa* spp.), Indian trumpet (*O. indicum*), and bitter bean (*P. speciosa*) in agricultural areas in southern Thailand (Sritongchuay et al., 2019; Stewart et al., 2014). These bats, particularly from the genus *Cynopterus*, are frugivorous, in which the most common component of their diet is fruit (Bumrungsri et al., 2007; Tan et al., 1998). Fruits are generally rich in energy but deficient in protein. Therefore, frugivorous bats are also known to visit flowering trees to consume floral parts and even leaves to fulfil their dietary requirements for protein (Rajamani et al., 1999).

Bats as Pollen Vectors in Agricultural Areas

Of the total 131 samples observed, 11 samples from *E. spelaea* and 14 from *C. brachyotis* were negative for pollen load. These 25 samples (19%) were thus excluded from further analysis.

A total of 12 pollen types were found on bat individuals with pollen load. These include *Durio* spp., *Sonneratia* spp., *C. pentandra*, *O. indicum*, *Elaeis guineensis* (oil palm), and six unidentified pollen types. In total, *E. spelaea* (n = 70) were found to carry the most significant number of pollen types which was 10 (1-5 pollen types per individual), followed by *C. brachyotis* (n = 34) with nine types (1-5 pollen types per individual). On the other hand, *Cynopterus horsfieldii* (n = 2) carried only *Musa* spp. and *Sonneratia* spp. pollen grains, which were also recorded for the other two bat species (Figure 3). Other than pollen grains of these two plant species, both *E. spelaea* and *C. brachyotis* were also found to carry five similar pollen types: *Durio* spp., *O. indicum*, *E. guineensis*, unknown pollen type A, and unknown pollen type B. The number (mean ± SE) of pollen types carried by *E. spelaea* (2.20 ± 0.13), *C. brachyotis*

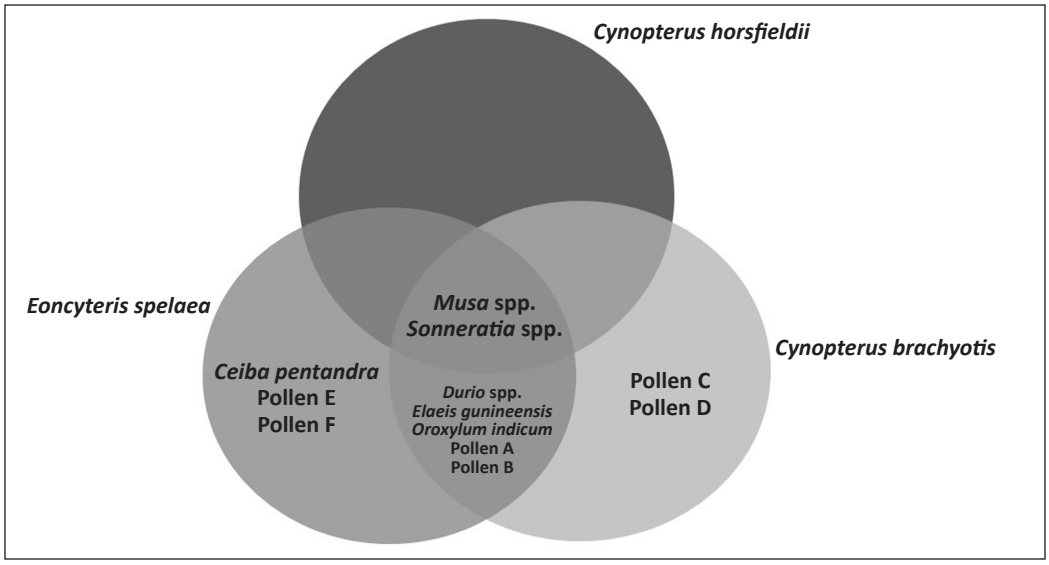

Figure 3. The Venn diagram of pollen types carried by the pteropodid bats caught at the Malaysian Agricultural Research and Development Institute (MARDI) Jerangau

(1.16 ± 0.17), and *C. horsfieldii* (1.50 ± 0.50) were found to be significantly different by the Kruskal-Wallis Test (H = 8.876, df = 2, p = 0.012), detected only between *E. spelaea* and *C. brachyotis*.

Results show that all bat species carried more than one pollen type on their bodies, indicating their visits to multiple tree species to fulfil their energy and nutrient requirements (Courts, 1998). Other than the chiropterophilous plants (*Musa* spp., *Durio* spp., *Sonneratia* spp., *C. pentandra*, *O. indicum*), pollen grains of oil palm (*E. guineensis*) were also recorded for *E. spelaea* and *C. brachyotis*. To our knowledge, oil palm has never been reported as a food source for pteropodid bats, even by Lim et al. (2018). Their study detected the plant materials occurring in the faeces of the pteropodid bats using DNA metabarcoding. These two bat species were reported to be the most common pteropodid bat species caught in the oil palm plantations in Malaysia (Mohd-Azlan et al., 2019; Syafiq et al., 2016), although no report indicated that they use oil palm plantations as a roosting site. Tan et al. (1997) reported that *C. brachyotis* could alter broad-leafed palms to form tents, while *E. spelaea* is a cave roosting species (Francis, 2008; Kingston et al., 2006). With oil palm pollen grains on the bats' bodies, it is unlikely that they roost in the oil palm plantation. Instead, they might collect the airborne pollen grains while manoeuvring through the plantations to get to the study site to forage for food.

Many chiropterophilous plants are of economic importance, highlighting a significant contribution of the pteropodid bats to human society. For example, *Durio* spp., *Parkia* spp., and *O. indicum* are commercial cash plants in Southeast Asia (Bumrungsri et al., 2013; Fujita & Turtle, 1991). On the other hand, *Sonneratia* spp. are exclusive mangrove trees (Tomlinson, 1986), playing an important role to protect coastal areas with their ability to reduce wave magnitude (Mazda et al., 2006). Thus, suggesting a high ecological importance of these bats to Terengganu, a state with a long coastline and large mangrove areas (Mohd Lokman & Sulong, 2001). Furthermore, in Terengganu, this genus was demonstrated to be mainly pollinated by pteropodid bats foraging at their flowers (Mohamed & Adzemi, 2017; Nor Zalipah et al., 2016), further emphasising the importance of these bats to the coastal communities.

From the ten pollen types observed for *E. spelaea*, pollen grains (mean ± SE) of *Durio* spp. were the most commonly found (344.29 ± 61.98), followed by *Musa* spp. (170.00 ± 49.11). These two pollen types were found to be significantly higher (Friedman's ANOVA, χ^2 = 248.62, df = 9, $p < 0.001$) in the numbers of pollen load recorded for this bat species as compared to other pollen types (Table 2). On *C. brachyotis*, the most commonly found pollen grains were *Musa* spp. (311.76 ± 87.01), which was significantly the highest in the number of grains among the nine pollen types (Friedman's ANOVA, χ^2 = 153.49, df = 8, $p < 0.001$). For *C. horsfieldii*, pollen grains of *Musa* spp. (350.00 ± 150.00) also recorded the highest in number, although the

Table 2
Number (mean ± SE) of each pollen type carried by the pteropodid bats caught at the Malaysian Agricultural Research and Development Institute (MARDI) Jerangau

	Eonycteris spelaea (n = 70)	*Cynopterus brachyotis* (n = 34)	*Cynopterus horsfieldii* (n = 2)
Durio spp.	344.29 ± 61.98[a]	41.18 ± 13.43[a]	-
Sonneratia spp.	14.29 ± 6.84[b]	2.94 ± 2.94[a]	50.00 ± 50.00
Ceiba pentandra	2.86 ± 2.86[b]	-	-
Musa spp.	170.00 ± 49.11[a]	311.76 ± 87.01[b]	350.00 ± 150.00
Oroxylum indicum	1.43 ± 1.43[b]	79.41 ± 79.41[a]	-
Elaeis guineensis	132.86 ± 124.22[b]	5.88 ± 4.10[a]	-
Pollen A	11.42 ± 3.83[b]	17.65 ± 7.87[a]	-
Pollen B	34.29 ± 14.80[b]	2.94 ± 2.94[a]	-
Pollen C	-	100.00 ± 94.03[a]	-
Pollen D	-	2.94 ± 2.94[a]	-
Pollen E	2.86 ± 2.01[b]	-	-
Pollen F	24.29 ± 11.41[b]	-	-

Note. Different superscript letters indicate significant difference ($p < 0.05$) between the pollen types from Kruskal-Wallis Test conducted for *E. spelaea* and *C. brachyotis*

difference in comparison to the other pollen types (*Sonneratia* spp.) was not statistically significant due to the small sample size (only two individuals).

Sonneratia spp. and *Musa* spp. were not only found on all the bat species captured in this study but pollen grains of *Musa* spp. were found in high numbers in all bat species. *Musa* spp. are steady-state plants (Stewart & Dudash, 2018), which bear a few flowers every day for several months (Heithaus et al., 1975), hence providing enough food resources for bats over extended periods. *Sonneratia* spp. in Setiu, Terengganu were found to flower year-round but with different peak flowering times between species (Nor Zalipah et al., 2020). Indeed, both *Sonneratia* spp. and *Musa* spp. are highly reliable food sources for bat species to forage at as compared to *Durio* spp. which showed the big-bang flowering strategy (Stewart & Dudash, 2018), in which plants flower massively only for a few days in a year (Gentry, 1974). The same finding was also reported by Thavry et al. (2017), in which pollen grains of *Sonneratia* spp. and *Musa* spp. were the main component in the diet of *E. spelaea* all year-round in Cambodia.

Other than *Musa* spp., Bumrungsri et al. (2013) concluded that *Parkia* spp. was the primary plant food source that provided pollen grains to *E. spelaea* continuously throughout the year in Thailand. However, pollen grains of *Parkia* spp. were not reported in our study for all bat species. During the peak flowering events of big-bang plants, pteropodid bats were found to switch their diets and utilise both the big-bang and steady-state plants (Bumrungsri et al., 2013; Stewart & Dudash, 2018; Thavry et al., 2017). The six unidentified pollen

types were not reported on bats' pollen load by Mohamed (2014) in the mangroves of Setiu, Terengganu. Nevertheless, the small number of grains recorded (except for Pollen C), contamination from airborne pollen was possible.

The pollen could also be from the 55 plant taxa listed by Lim et al. (2018) as an essential food source for the pteropodid bats in Peninsular Malaysia. However, no source of the plant parts (whether the plant materials in the faeces were pollen grain, seed, flower parts, and leaves) identified in the study was provided. Thus, we could not confirm the identification of the six unidentified pollen types recorded in our study. As digested pollen grains were generally corresponding to the pollen loads on the bats' bodies (Bumrungsri et al., 2013), foraging at the flower thus may result in the pollination of the flowers (Nor Zalipah et al., 2016; Stewart & Dudash, 2017). Nevertheless, the bats' function as pollen vectors in the agricultural areas should not be overlooked. With their high mobility (Horner et al., 1998; Marshall, 1983), pollen dispersal by bats will affect the genetic structure of the plant community, and hence has significant evolutionary consequences (Fleming & Kress, 2013).

Bats as Pollinating Agents of Durian Trees

Durian pollen grains were recorded on only two bat species and were not found on *C. horsfieldii*. However, from the total captured, 54% (38 individuals from the total 70) of *E. spelaea* and 23% (eight individuals from the total 34) of *C. brachyotis* individuals were found with the conspecific pollen grains. Of these, five (7%) and two (6%) individuals of the former and latter species respectively were carrying only the conspecific pollen grains on their bodies at the time of their capture.

For these two bat species, however, the number of heterospecific pollen grains on the bats' bodies was higher than the conspecific pollen grains at the time of their capture (Figure 4). For *E. spelaea* the number (mean $\pm$ SE) of conspecific pollen grains was only 211.43 ± 47.09 as compared to 527.14 ± 136.00 grains of heterospecific pollen (Wilcoxon signed-rank test, $T = 573.50$, $p = 0.001$). *Cynopterus brachyotis* recorded 532.35 ± 152.01 heterospecific pollen grains, notably higher than the 32.35 ± 11.73 conspecific pollen grains (Wilcoxon signed-rank test, $T = 533.00$, $p < 0.001$). *Eonycteris spelaea*, however, was found to carry a significantly higher number of conspecific pollen grains (211.43 ± 47.09) as compared to *C. brachyotis* (32.35 ± 11.75) as detected by the Mann-Whitney test conducted ($U = 762.50$, $p = 0.001$).

Conspecific pollen load on the bodies of flower visitors was recently proven to be a strong indication of the pollen transfer to the stigma of the flowers to initiate pollination (Stewart & Dudash, 2017). We reported more individuals of *E. spelaea* carrying conspecific pollen grains than *C. brachyotis*. The number of the conspecific pollen grains was also significantly higher than the latter bat species. Hence *E. spelaea* was a more important pollinating agent

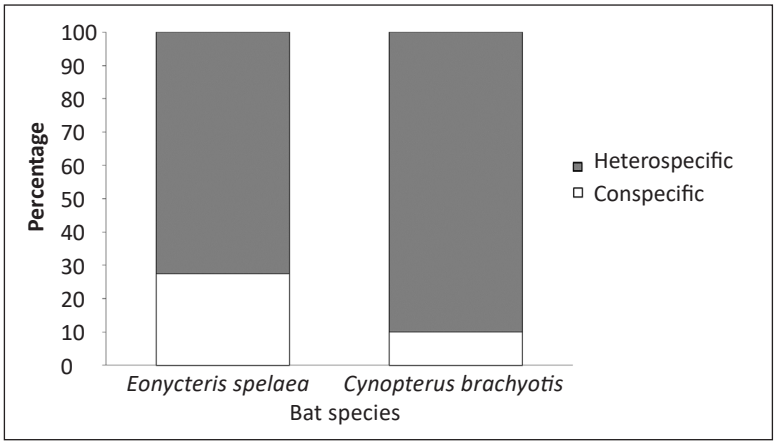

Figure 4. Composition of conspecific and heterospecific pollen grains observed for *Eonycteris spelaea* (n = 70) and *Cynopterus brachyotis* (n = 34) caught at the Malaysian Agricultural Research and Development Institute (MARDI) Jerangau

for durian than *C. brachyotis*. *Eonycteris spelaea*, as nectarivorous bats, was also touted to be the more important pollinating agent as compared to other frugivorous pteropodid bats (such as *C. brachyotis* and C. *horsfieldii*) foraging at *D. zibethinus* in agricultural areas in southern Thailand (Stewart & Dudash 2017; Stewart et al. 2014). Thus, not only *E. spelaea* visited the flowering durian trees significantly more often than the frugivorous bats, but the former also carried significantly more conspecific pollen grains on their bodies than the latter. For the frugivorous bats visiting flowering trees to forage for floral resources such as *Rousettus leschenaultii* (Leschenault's rousette) high visitation compensates for their low conspecific pollen load, thus also providing reliable pollination service to the trees they visited (Stewart & Dudash, 2017).

Another study has also reported *E. spelaea* as the principal pollinating agent of *D. zibethinus* in agricultural areas of southern Cambodia (Thavry et al., 2017). A similar finding was also reported on semi-wild durian, which is sparsely distributed in secondary forests in managed agroforest areas in Sulawesi, Indonesia (Sheherazade et al., 2019). In that study, two larger pteropodid bats, *Pteropus alecto* (black flying fox) and *Acerodon cebelensis* (Sulawesi flying fox), were also pollinating agents of the durian trees. However, their visitation frequencies to the flowers were lower than those recorded by *E. spelaea*. Other studies in Peninsular Malaysia by Aziz et al. (2017) reported that a giant pteropodid bat on Tioman Island, the island flying fox (*Pteropus hypomelanus*), was a more effective pollinating agent for *D. zibethinus* as compared to *E. spelaea*. However, this giant pteropodid bat is confined only to islands in the Indo-Australian region (Francis, 2008). Thus, it is not present in the study area of the present study. Furthermore, compared to the small pteropodid bats, the two flying foxes recorded in Malaysia,

Pteropus hypomelanus and *Pteropus vampyrus* (large flying fox), are protected under the Wildlife Conservation Act (2010). Hence, less attention is given to the small pteropodid bats in Malaysia, probably due to underestimated economic and ecological functions. The information gain from this study nevertheless has contributed to a greater understanding of the importance of these small pteropodid bats.

CONCLUSION

Three small pteropodid bats, namely, *Cynopterus brachyotis*, *Cynopterus horsfieldii*, and *Eonycteris spelaea*, were captured when visiting flowering durian trees (*Durio zibethinus* and *Durio lowianus*) at agricultural areas in Hulu Terengganu. All three species were found to carry pollen grains on their bodies. *Cynopterus brachyotis* and *E. spelaea* carried multiple pollen types on their bodies, thus indicating their essential role as pollen vector and pollinating agents in the study area. *Cynopterus horsfieldii,* on the other hand, with only two individuals caught, recorded two pollen types, not including durian pollen grains. For the other two bat species, *E. spelaea* was likely to be a more important pollinating agent of durian than *C. brachyotis*. Not only was *E. spelaea* frequently captured near the flowering trees, but the majority of the captures were also found to carry a significantly high number of conspecific pollen grains on their bodies. High conspecific pollen load may contribute to the high potential of pollen transfer from the bats' bodies to the stigma of the durian flowers they forage at, resulting in the pollination of the flowers. Hence, the small pteropodid bats in agriculture areas have high conservation value due to their essential role in pollinating the cash crop durian trees.

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Review article

Genome Editing for the Development of Rice Resistance against Stresses: A Review

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ABSTRACT

Food security is the most crucial issue faced by humans considering the rising population. Rice, a staple food consumed by nearly 50% of the world's population, faces challenges to meet the consumers' demand to ensure self-sufficiency amidst various abiotic and biotic stresses. Drought, salinity, heat, and infection by bacteria and viruses are the main challenges in rice cultivation. Genome editing technology provides abundant opportunities to implement selective genome modifications. Moreover, it finds the functional implications of different genome components in rice and provides a new approach for creating rice varieties tolerant of stresses. This review focuses on rice production worldwide and challenges faced in rice cultivation, and current genome editing tools available that can be utilised for crop breeding and improvement. In addition, the application of genome editing to develop biotic and abiotic resistance rice varieties is critically discussed.

Keywords: Abiotic stress, biotic stress, genome editing, rice

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INTRODUCTION

For years, plant breeders have used conventional breeding to produce improved crops with desirable traits such as resistance to pests and diseases, enhanced quality and yield, disease resistance, shortened growing seasons, high nutritional content, extended shelf life, and better adaptation to various topographies. However, the conventional

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breeding method gives unpredictable results. Therefore, a more extended period is required to achieve crops with desirable traits. Nowadays, breeders use the molecular breeding method to assist the conventional approach and increase the likelihood of success in breeding programs. In addition, the molecular breeding method reduces time, cost, and workloads compared to the conventional breeding method. There are various branches of molecular breeding for crop improvement study, such as genome editing.

Genome editing is an effective tool for introducing mutations in plants, and the results showed room for trait improvements. Gene editing is a precise and efficient advanced molecular biology technique with targeted modifications at genomic loci (J. Zhang et al., 2018). Examples of genome editing tools are zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/ CRISPR-associated system (CRISPR/Cas) (Boettcher & McManus, 2015). Using DNA sequence-specific manipulations, many studies shows success in producing desirable traits crops via genome editing.

Global Rice Cultivation and Production

Out of 300,000 edible plant species, only 0.7% is regularly consumed for their nutritional values (Voss-Fels et al., 2019). However, essential commodities contributed to the average global production, including fruits (10%), vegetables (13%), sugarcane (23%), roots and tubers (11%), and oil-bearing crops (11%). In addition, cereal crops such as rice, wheat, and maize are considered essential crops, providing 60% of energy in the human diet.

Apart from wheat, rice is rendered the most vital edible crop in the world. Rice is consumed by more than 3.5 billion globally and is an important income source for families in developing countries. The average global rice production was recorded at 750 million tonnes (Food and Agriculture Organization [FAO], 2020). Thus, the overall area for rice cultivation continuously increases to fulfil a rising global rice demand following the rapid population growth. From 1994 to 2018, the total rice yields increase from 5.39 million tonnes to 7.82 million tonnes. The Asia regions consume 80% of the rice production. Referring to Figure 1, Asian countries such as China, India, Indonesia, Bangladesh, and Vietnam are the world's largest rice producers and consumers (FAO, 2020). However, rice yield loss due to abiotic, biotic stresses, and other factors cause pressure to improve rice yield and quality to ensure self-sufficiency. Therefore, rice quality improvement must be realised to overcome current agriculture issues and improve rice production and farmers' incomes to achieve the zero hunger goal by 2030.

Challenges Faced in Rice Cultivation

Based on the latest United Nations population prospects, the world population is predicted to increase by 34%, from 7.6 billion today to 10 billion in 2050 (Voss-Fels et al., 2019). The current rice

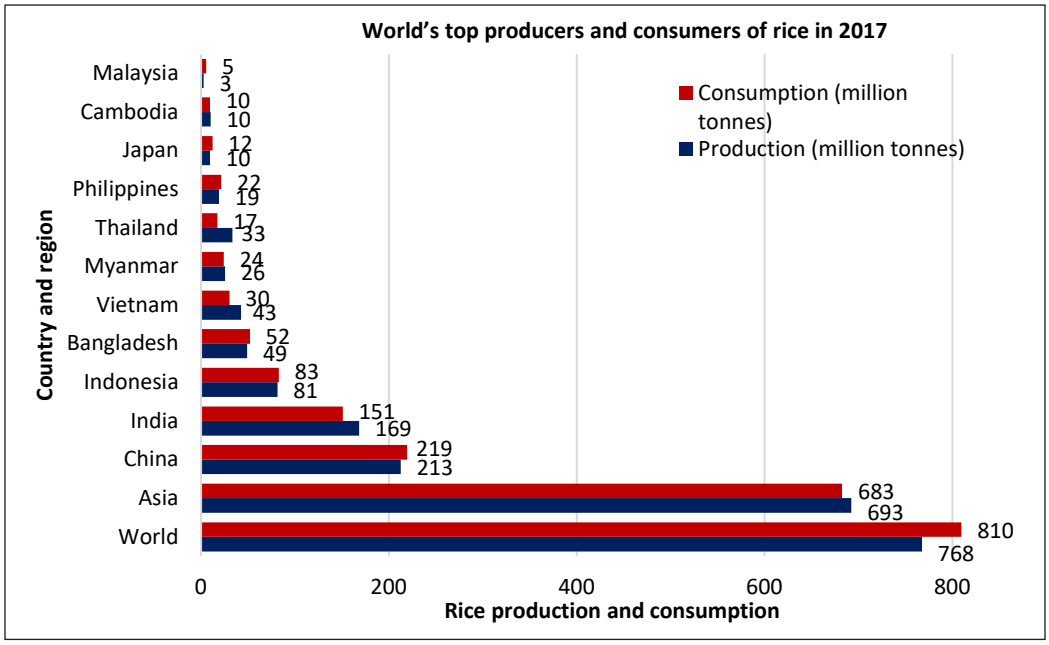

Figure 1. World's top producers and consumers of rice, by region and country (FAO, 2020)

production is low and unlikely to meet the predicted needs and demands in 2050 (Ray et al., 2013). Therefore, the growing global human population may bring challenges in achieving global food security.

Global warming negatively impacted the yield, making it alarming as Earth's atmosphere can influence agricultural activities. Rapid climate changes may initiate food insecurity on a global scale. Recent studies explore the relationship between the ozone decline and other stresses on soybean, maize, wheat, and rice yield globally (Mills et al., 2018). The authors also found that soybean is the most sensitive towards ozone reduction, followed by wheat, maize, and rice. Ozone decline reduces the annual global yield on soybean (12.4%), wheat (7.1%), rice (4.4%), and maize (6.1%) based on stomatal uptake. It was also reported that India, Bangladesh, China, and Indonesia were severely affected due to the ozone decline.

Extreme weathers such as drought can inhibit crop production and yield quality. Over the last three decades, drought effects on rice yield were observed in several countries. Bangladesh losses of 2 million tonnes of rice in 1978-1979 due to the seasonal drought phenomenon. In 2009, Bangladesh was devastated with a 50-60% yield reduction (Climate Change Cell, 2009). About 40% yield losses were reported in several eastern Indian regions and 20% yield losses in Thailand due to extreme drought conditions in 2004 (Pandey & Bhandari, 2009). The direct effect of drought on the rising temperature is concerned as the rice crops are temperature-sensitive. The rising temperature affects the flowering and grain filling stage, inhibiting the rice yield (Alam et al., 2012). With every 2°C temperature

rise, the rice yield decreases from 5 million tonnes per hectare to 4 million tonnes per hectare (Alam et al., 2012). Recent studies in the Mun River Basin, Thailand, rice cultivation area showed that yield decreases between 2% to 10% per 1°C increment. With every 1°C increase in the dry season, the rice yield decreases by 10% (Prabnakorn et al., 2018). Apart from drought and rising temperature, floods can also be equally devastating to rice-producing countries (Alemu & Assaye, 2020; Ettang, 2020).

Salinity stress is another major constraint in rice cultivation since rice is a salt-sensitive crop. Salinity stress in rice had various effects on the growth and development stages of rice. Rice during the flowering stage is most sensitive to salt stress that inhibits the grain yield (Clermont-Dauphin et al., 2010). Salt stress can also reduce the number of tillers, grain weight, and panicle length. Most rice producers in Southeast Asia, such as Vietnam, Thailand, and Bangladesh, face salinity stress problems, especially in areas exposed to seawater (Clermont-Dauphin et al., 2010).

The climates and environmental threats have become substantial, notably during unpredictable extreme weather. Drastic reduction of rice as a staple food will cause mass starvation. In addition, small farmers are at risk of poverty as they lose their source of income or are displaced due to natural disasters like floods (Ettang, 2020). Overcoming or adapting to these challenges will be too overwhelming and challenging for the farmers. Besides, global rice cultivation is at risk of disease from bacteria, fungi, viruses, and pests. Notable diseases that affect global rice production are a blast, sheath blight, and sheath rot caused by fungi infection, bacterial blight disease, and rice tungro disease caused by viral infection (Bunawan et al., 2014; Gnanamanickam, 2009).

Genome Editing Tools

Genome editing technology has been improved rapidly in the last two decades to overcome the shortcomings of conventional breeding methods for future crop improvement. Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) are the example of the technology using the engineered site-specific nucleases (SSNs) (Mishra et al., 2018). Owing to the high efficiency and specific mutation at the desired target requiring RNA-guided DNA nucleases, the clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease 9 (CRISPR/Cas9) system is the most advanced system using protein-DNA interaction. New advanced and improved genome editing tools were created based on the previous system, clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease 12a (CPISPR/Cas 12a), base editing and prime editing in that order (Figure 2).

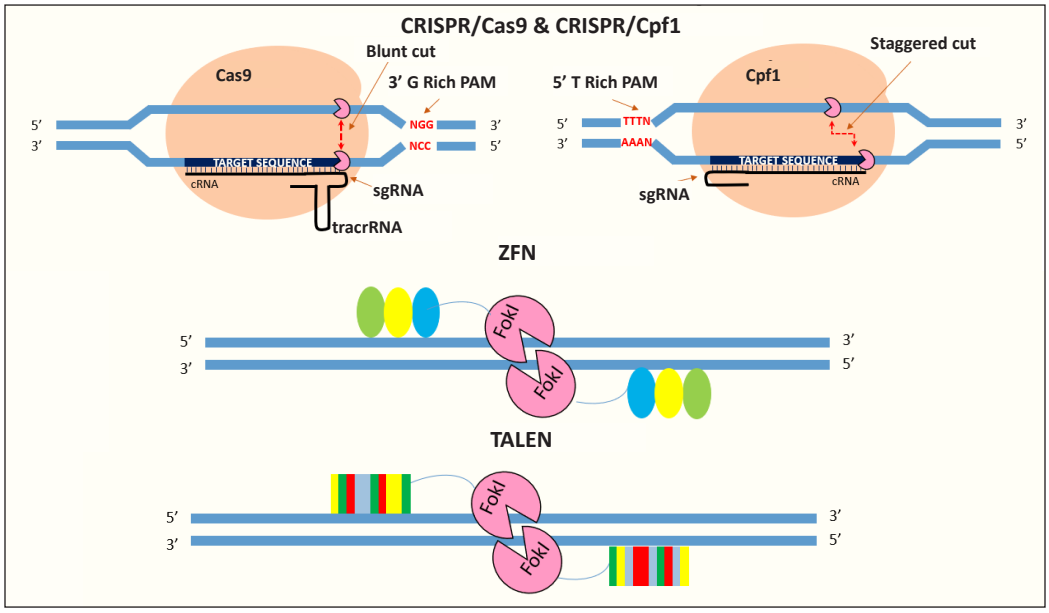

Figure 2. Schematic illustrating the engineered type II CRISPR-Cas9, CRISPR-Cpf1, ZFN, and TALEN system. More recently, the use of clustered regularly interspaced short palindromic repeats (CRISPR) vectors has provided a 'breakthrough' in the technique of specific genome editing to develop mutants
Note. CRISPR/Cas9 = Clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease 9; CRISPR/Cpf1 = Clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease in *Prevotella* and *Francisella* 1; ZFN = Zinc finger nuclease; TALEN = Transcription activator-like effector nuclease

Zinc Finger Nucleases (ZFNs). ZFNs are generated from mergers of zinc fingers (ZFs) domain linked to *Fok*I endonuclease (Urnov et al., 2010). It is a non-specific restriction enzyme and does not involve sequence recognition (Pernstich & Halford, 2012). Another half of the ZFs fused to the *Fok*I endonuclease is needed for the DNA cleavage domain to be active and cut at a specific DNA sequence. The ZFs recognise and bind to three specific nucleotides. When the ZFN occurs as a dimer, the 3-4 ZFs arrays recognise and bind to the 18-24 bp nucleotides (Petolino, 2015). Nevertheless, some reports that ZFNs exhibited off-target cleavage and improved ZFNs efficiency and precision have been made. Researchers were able to multiply the turnover rate of the ZFN endonuclease by designing variants like sandwiched nucleases attached to one or two artificial zinc-finger proteins (APZs) and adding a single-chain *Fok*I dimer to the complex (Mino et al., 2009).

Transcription Activator-Like Effector Nucleases (TALENs). Like ZFNs, TALENs involve fusing the *Fok*I endonuclease and the 34 amino acids DNA binding repeat domain (M. Zhang et al., 2014). The *Fok*I endonuclease dimerisation with another half of the TALEN will mediate DNA cleaving. The transcriptional activator-like effectors (TALE) are the DNA binding domain for TALENs. TALENs can facilitate one-to-one

pairing of nucleotides, more precise than ZFNs. Moreover, TALENs can achieve sequence specificity in contrast to ZFNs due to their sequence-specific DNA binding domain, derive from proteins excreted by *Xanthomonas* (Joung & Sander, 2013). The DNA specific binding occurs at positions 12 and 13 of each repeat (Mahfouz et al., 2011). Hence, TALENs are more beneficial than ZFNs in terms of protein guided genome editing. TALENs are also less toxic to the cells due to their improved specificity and reduced off-target sites cleavage.

CRISPR/Cas9. CRISPR/Cas9 system involves RNA-DNA interaction to achieve sequence specificity. It is more flexible, reliable, and cost-effective compared to ZFNs and TALENs. The CRISPR system was first discovered in *Escherichia coli* and its role in bacterial resistance to viruses (Doudna & Charpentier, 2014; Jinek et al., 2012). CRISPR/Cas9 can site-specific binding by combining the system with endonuclease Cas9 and utilised in different non-bacterial organisms (Kotwica-Rolinska et al., 2019; Markossian & Flamant, 2016). CRISPR/Cas9 mechanism begins with the site-specific binding of a ~100-nt sequence single guide RNA (sgRNA) to the target sequence of a 5'-NGG-3' protospacer adjacent motif (PAM). The trans-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) of the sgRNA was designed to assist sequence-specific Cas9 double -stranded DNA (dsDNA) cleavage (Jinek et al., 2012). A CRISPR complex will form and initiate cleaving of the target DNA upon binding, creating a double-strand DNA break (DSBs). CRISPR/Cas9 adopts the homology-directed repair (HDR) while repairing the DSBs. In recent years, CRISPR/Cas9 technology has been applied in rice cultivation for functional analysis and crop improvement studies (Ren et al., 2019; Schachtsiek & Stehle, 2019).

CRISPR/Cas12a. CRISPR/Cas12a is the advanced genome editing tool derived from CRISPR. The CRISPR-associated endonuclease in *Prevotella* and *Francisella* 1 (Cpf1) or Cas12a is an RNA endonuclease, similar to Cas9 nuclease (Zetsche et al., 2017). CRISPR/Cas12a is more beneficial and cheaper than CRISPR/Cas9 system despite the similarity in its mechanism. The synthesised sgRNA attached to the Cas12a nuclease only requires crRNA (~42-nt), enhancing the delivery speed owing to its smaller sgRNA-Cas12a complex (Zetsche et al., 2017). The Cas12a is more versatile than Cas9 as it can prepare the pre-crRNA to crRNA and simultaneously cleaves the DNA double-strand (Nakade et al., 2017). Unlike Cas9, Cas12a recognises T-rich (5'-TTTN-3 or 5'-TTN-3') PAM sites and cleaves DNA at 23-17 bp downstream of the PAM sites generating staggered 5' overhangs. These features prevent the PAM site's disruption, allowing flexibility in choosing the target site, minimising the non-specific mutations at the cut site, and enabling multiplexed genome editing using one vector (S. Li et al., 2019; Zetsche et al., 2017). Within a year of the first report of Cas12a advantages properties, many publications demonstrated its specificity in maize, *Arabidopsis*, rice, and other plants (Malzahn et al., 2019).

Base Editing (BE). BE technology was developed to edit individual base pairs in DNA using base editors. BE can correct single base pairs instead of cutting and replacing the entire DNA sequence, such as CRISPR (Komor et al., 2016). It is commonly used to fix point mutations in the DNA. In addition, it is often applied to correct disease-related mutations in the human genomes (Liang et al., 2017; Zhao et al., 2020). The base editors execute BE consisting of three proteins that can inspect and bind to the target DNA sequences, convert C base to T base via chemical reaction, and prevent the edited T base from being removed. The permanent transition of C-G-to-T-A and A-T-to-G-C can be performed by the cytosine base editors (CBEs) and adenine base editors (ABEs), respectively. The two base editors were engineered together to fuse with a Cas9 nickase (nCas9) and successfully applied in plants to correct single-base changes responsible for phenotypic variations (Zong et al., 2018). As multiple cytosines and adenine replacement are present, unwanted bystander edits may occur, making BE less precise.

Prime Editing. Prime editing is the latest genome editing technology derived from yeast and mammalian cells (Anzalone et al., 2019). Prime editing can edit the genome precisely without using DSBs and a donor DNA template. Unlike BE, prime editing can conduct any base-to-base conversion where modification is limited to C-G-to-T-A and A-T-to-G-C. The technology required an engineered prime editing guide RNA (pegRNA) with a spacer sequence and a prime editor (PE). The spacer sequence is complementary to a primer binding site (PBS) sequence; a reverse transcriptase (RT) template consist of desired edit sequence and one strand of the DNA. In contrast, the PE consists of RT enzyme and Cas9 nickase (Cas9n). Genome editing via prime editing involves reverse transcription. The pegRNA and PE will form a complex and bind to the complementary DNA strand and RT template guided by the pegRNA. Afterwards, Cas9n cuts the PAM- containing DNA strand, generating a flap, and the PBS binds to the 3' cut strand. The RT triggers integrating the desired edit sequence from the RT template region to the PAM-containing DNA strand via reverse transcription. The process produced two redundant single-stranded DNA flaps (the unedited 5' and edited 3' DNA flaps). The edited DNA strand will be integrated into the genome at the cut DNA strand, and the Cas9n will remove the unedited DNA strand. Stable incorporation of the edited sequences into the genome will occur following the repair of the heteroduplex DNA via a cellular DNA repair mechanism. Prime editing can produce multiple and precise nucleotides replacement, unlike base editing, which is incompetent when multiple adenine and cytosine replacement is present (Abdullah et al., 2020). Prime editing technology has a much lower target editing than Cas9 due to the additional two hybridisation steps between DNA and pegRNA and DNA-reverse transcript

templates. Unlike PAM-sequence, prime editing does not require a suitable distance to initiate, making the target scope more flexible. The application of prime editing in plants is limited due to its new technology. However, numerous studies have been conducted to optimise the prime editing tools used for crop improvement. Nevertheless, the recent application of prime editing in plants reported low editing efficiency. C. Lin et al. (2020) reported that prime editing in wheat and rice could generate transversion, substitutions, insertions, and deletions despite lower editing efficiency than base editing. Butt et al. (2020) and R. Xu et al. (2020) also reported low editing efficiency despite being capable of editing at different genome sites and nucleotide substitutions in plants. Thus, plant prime editing is much more flexible and versatile though further optimisation is needed.

Rice Improvement via Genome Editing

The availability of target site-specific mutations or base editing allows researchers to utilise genome editing tools to improve rice cultivation. Extensive studies have been conducted on genome editing in rice and its effect on crop productivity and development, such as metabolisms and stress responses.

Resistance against Abiotic Stress. Abiotic stresses are the severe development pressures predicted to deteriorate with anticipated climate change (Pereira, 2016). Many studies have focused on understanding the plants' molecular basis reaction with the environmental factors in recent decades. Over a few decades, approximately 100 genes are identified in rice, contributing to abiotic stress response (F. Wang et al., 2016). Various methods, such as identifying multiple genes/pathways and regulatory networks implied in stress responses, have been solved. The growing production of abiotic stress-tolerant rice demonstrates a successful crop yield improvement via genome editing (Table 1).

Drought. Regarding the importance of abiotic stress, it was proven that deactivation and degradation of the mediator of OsbZIP46 deactivation and degradation (MODD) protein mediate (OsbZIP46) inhibit ABA signalling, increasing the rice resistance to drought. This method can enhance drought tolerance in rice plants by producing MODD knock out mutants (Tang et al., 2016). Liao et al. (2019) reported that changing leaf physiology like rolled leaf genotype reduces the water loss rate, enhancing drought tolerance response in rice. Through CRISPR/Cas system, *SEMI-ROLLED LEAF 1* (*SRL1*) and *SEMI-ROLLED LEAF 2* (*SRL2*) rice mutants increase drought tolerance and improved survival rate during the seedling stage. CRISPR/Cas9 mediated knockout of *OsmiR535* study showed that *Osmir535* mutant enhanced rice seedlings survival rate after dehydration stress, suggesting its potential as genetic editing target for drought tolerance (Yue et al., 2020).

Table 1
The improved trait of genome editing technology for the development of stress-tolerant rice varieties

Application	Genome editing tool	Targeted gene	Reference
Drought tolerance	CRISPR/Cas9	*OsMODD*	Tang et al. (2016)
	CRISPRi	*OsNAC14*	Shim et al. (2018)
	CRISPR/Cas9	*SRL1*, *SRL2*	Liao et al. (2019)
	CRISPR/Cas9	*OsAAA-1*, *OsAAA-2*	Lu et al. (2020)
	CRISPRa dCas9	*AREB1*	Paixão et al. (2019)
	CRISPR/Cas9	*OsmiR535*	Yue et al. (2020)
Salinity tolerance	CRISPRi	*OsSIT1*	C.-H. Li et al. (2014)
	CRISPR/Cas9	*OsmiR535*	Yue et al. (2020)
	CRISPR/Cas9	*OsRR9*, *OsRR10*	W.-C. Wang et al. (2019)
	CRISPR/Cas9	*OsRR22*	A. Zhang et al. (2019)
Cold tolerance	CRISPR/Cas9-HDR	*OsCTB4α*	Z. Zhang et al. (2017)
	CRISPR/Cas9-HDR	*OsCOLD1*	Y. Ma et al. (2015)
	CRISPR/Cas9	*OsPIN5b*, *GS3*, and *OsMYB30*	Y. Zeng et al. (2020b)
Bacterial blight resistance	TALEN	*OsSWEET14*	Blanvillain-Baufumé et al. (2017)
	TALEN	*OsSWEET11*, *OsSWEET14*	Z. Xu et al. (2019)
	CRISPR/Cas9	*OsSWEET14*	Zafar et al. (2020)
	TALEN	*Os11N3* (*OsSWEET14*)	T. Li et al. (2012)
	CRISPR/Cas9	*OsSWEET14*	X. Zeng et al. (2020a)
	CRISPR/Cas9	*SWEET11*, *SWEET13*, *SWEET14*	Oliva et al. (2019)
	CRISPR/Cas9	*Os8N3* (*OsSWEET11*)	Kim et al. (2019)
	CRISPR/Cas9	*Xa13*	C. Li et al. (2020)
	CRISPR/Cas9	*TMS5*, *Pi21*, and *Xa13*	S. Li et al. (2019)
Bacterial leaf streak resistance	TALEN	*Os09g29100*	Cai et al. (2017)
Rice blast resistance	CRISPR/Cas9	*OsERF22*	F. Wang et al. (2016)
	CRISPR/Cas9	*OsSEC3A*	J. Ma et al. (2018)
Rice tungro resistance	CRISPR/Cas9	*eIF4G*	Macovei et al. (2018)

Note. CRISPR/Cas9 = Clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease 9; CRISPRi = Clustered regularly interspaced short palindromic repeats interference; CRISPRa dCas9 = Clustered regularly interspaced short palindromic repeats activation defective CRISPR-associated endonuclease 9; CRISPR/Cas9-HDR = Clustered regularly interspaced short palindromic repeats/CRISPR-associated endonuclease 9-homology directed repair; TALEN = Transcription activator-like effector nuclease; *OsMODD = Oryza sativa mediator of OsbZIP46 deactivation and degradation*; *OsNAC14 = Oryza sativa Nascent polypeptide-Associated Complex (NAC) domain-containing protein 14*; *SRL1 = SEMI-ROLLED LEAF 1*; *SRL2 = SEMI-ROLLED LEAF 2*; *AREB1 = Abscisic acid-responsive element binding protein1*; *OsSIT1 = Oryza sativa salt intolerance 1*; *OsRR9 = Oryza sativa response regulator 9*; *OsRR10 = Oryza sativa response regulator 10*; *OsRR22 = Oryza sativa response regulator 22*; *OsCOLD1 = Oryza sativa chilling tolerance divergence 1*; *OsPIN5b = Oryza sativa PIN protein 5B*; *GS3 = Grain size 3*; *OsMYB30 = Oryza sativa Myb transcription factor 4 paralog*; *Xa13 = Xanthomonas oryzae* pv. *oryzae resistance 13*; *OsERF22 = Oryza sativa ethylene response factor 22*; *OsSEC3A = Oryza sativa subunit of the exocyst complex 3A*; *eIF4G = Eukaryotic translation initiation factor 4 gamma 1*

Salinity. Plant abiotic stress tolerance can be easily improved by generating CRISPR-Cas9-mediated knockout/knockdown of harmful regulatory genes. It is observed via editing of *Oryza sativa SRFP1* and *Oryza sativa response regulator 22* (*OsRR22*). The *SRFP1* is a negative salinity tolerance gene where new mutants produced via CRISPR/Cas9 will increase salinity tolerance (Fang et al., 2015). A. Zhang et al. (2019) reported that *OsRR22*-induced mutations enhanced salinity tolerance compared to wild-type plants during the seedling stage.

Cold. Cold stress can majorly affect the growth and rice yield. A recent study identified *Oryza sativa Myb transcription factor 4 paralog* (*OsMYB30*) as a gene associated with cold tolerance (Y. Zeng et al., 2020b). The *OsMYB30* Nipponbare mutants demonstrated enhanced cold tolerance compared to wild type.

Resistance against Biotic Stresses. Rice is susceptible to various biotic stresses resulted in poor productivity and quality. Biotic stresses such as insect pests, fungi, bacteria, and nematodes (Singh et al., 2020) can cause devastating diseases such as rice blight, rice tungro, rice blast, and rice sheath rot (Gnanamanickam, 2009). Strategies to mitigate rice diseases include chemical control, varietal resistance breeding, biological control, cultural practice, and genetically modified plants with disease resistance traits (Abo & Sy, 1997). Numerous studies have successfully developed rice resistance towards biotic stress diseases by applying and understanding advanced genome editing tools such as CRISPR.

Bacterial Blight. Bacterial blight in rice is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), a Gram-negative bacterium (Niño-Liu et al., 2006). Attempts on enhancing rice resistance towards blight disease were conducted by editing the promoter of the *SWEET* gene family (Blanvillain-Baufumé et al., 2017; Z. Xu et al., 2019; Zafar et al., 2020). The transcription activator-like effectors (TALEs) target promoter effector-binding elements (EBEs) of *SWEET11*, *SWEET13*, and *SWEET14* belonging to *SWEET* family clade III (Yang et al., 2006) when the plants are infected with *Xoo*. Since *SWEET* genes encode for sugar efflux transporters, induction of these genes hijacks the sugar export into the apoplast, fulfilling the nutritional requirement of the pathogen (Cohn et al., 2014). Besides that, bacterial blight-resistant rice can be achieved by editing the *Xanthomonas oryzae* pv. *oryzae resistance 13* (*Xa13*) promoter via CRISPR/Cas9 (C. Li et al., 2020). The *Xa13* gene in rice is a fully recessive resistance allele of *Os-118N3*, a disease-susceptibility gene against bacterial blight (Antony et al., 2010). Multiplex gene editing of *TMS5*, *Pi21*, and *Xa13* via CRISPR/Cas9 can enhance the rice resistance to blight disease (S. Li et al., 2019).

Bacterial Leaf Streak. Bacterial leaf streak (BLS) is a widespread disease in rice-growing regions globally, causing 10% to 20% yield reduction and 40% yield loss

(Niño-Liu et al., 2006). However, BLS can be mitigated by enhancing the resistance to *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) using TALEN editing of the Tal7 binding site of the *Os09g29100* gene promoter (Cai et al., 2017). Tal7 binds to two EBE sites of *Os09g29100* promoter, which encodes for *cyclin-D4-1* and activation of *cyclin-D4-1* suppressing the *avrXa7-Xa7* mediated rice's defence.

Rice Blast. Rice blast disease is caused by the fungus *Magnaporthe oryzae* (Couch & Kohn, 2002), leading to a 10% to 30% loss in rice production annually (Sakulkoo et al., 2018). Genome editing tools can effectively enhance rice resistance against rice blast disease. Targeted mutation of ERF transcription factor gene, *Oryza sativa ethylene response factor 22* (*OsERF22*) via CRISPR/Cas9, can enhance rice blast resistance in its knockout mutants (F. Wang et al., 2016). The mutant plants contained insertion or deletion mutations at the target site while maintaining the agronomic traits compared to the wild-type plants. Meanwhile, disruption of the *OsCE3A* by CRISPR/Cas9 demonstrates enhanced resistance to *M. oryzae* alongside the increased level of salicylic acid. *Oryza sativa* subunit of the exocyst complex 3A (OsSEC3A) is a subunit of the exocyst protein complex that interacts with OsSNAP32 protein involved in rice blast resistance (J. Ma et al., 2018; Mishra et al., 2021).

Rice Tungro. Rice tungro disease (RTD) is caused by the combination of two distinct viruses, *Rice tungro spherical viruses* (RTSV) and *Rice tungro bacilliform viruses* (RTBV) (Hibino et al., 1979). This disease frequently occurs in South and Southeast Asian countries (Bunawan et al., 2014), causing a $ 1.5 billion loss annually (Dai & Beachy, 2009). IR64 rice variety is known to be susceptible to RTD (Macovei et al., 2018). However, using CRISPR/Cas9, *IR64* mutant of the *eukaryotic translation initiation factor 4 gamma 1* gene (*eIF4G*), demonstrating resistance to RTD.

CONCLUSION

Rice production faces various challenges and threats, mainly climate change, abiotic, and biotic stressors leading to unsustainable development and yield losses. Genome editing has been used to address the limitations of conventional breeding methods in developing high yield and quality rice varieties to meet the growing consumer demands. The genome editing tool is cost-effective, accurate, time-saving, reliable, and robust compared to the conventional methods. However, several aspects need to be reviewed to utilise the tools properly. Low editing efficiency issues such as undesired off-target events/ mutations can be solved by developing high precision guide RNAs and understanding improved Cas9 variants. CRISPR technique requires optimum plant tissue culture and transformation systems. However, both systems depend on the species, genotype, and challenging commercial rice varieties where the systems are not optimised.

Moreover, further data validation is needed on genome-edited rice trait improvement in both controlled and field environments to understand the potential effects on the rice in the natural environment. Field trials and observation is crucial to evaluate the genome-edited plants' performance in the natural environment. Ethical and public acceptance of genome editing over transgenic methods needed to be addressed. Typically, genome-edited crops are associated with genetically modified organisms (GMOs) issues. Debates on the uncertain safety and process of these products cause public unrest and rejection of genome-edited crops. Education among the public of its safety and advantages is needed, especially in Asian countries where rice cultivation is dominant and threatened by abiotic or biotic stresses. Preserving the effective disease resistance plants developed through genome editing over time also poses a tremendous challenge. Future studies on multiplex genome editing strategies to target multiple disease resistance trait genes are desirable. Genome editing tools will likely transform the future of crop improvement and achieving zero hunger goals by securing feed for a growing population.

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Diversity, Composition, Taxa Biomarkers, and Functional Genes of Fish Gut Microbes in Peat Swamp Forests and its Converted Areas in North Selangor, Malaysia

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ABSTRACT

The aquatic organisms in peat swamp forests are under threat due to habitat degradation resulting from human activities. This study determines the fish gut microbes' diversity, composition, taxa biomarkers, and functional genes in peat swamp forests and its converted areas in North Selangor, Malaysia. Three undisturbed and disturbed areas nearby the peat swamp forests were selected. First, the 16S amplicon metagenomic analysis was conducted to assess the composition and diversity of bacterial communities in fish gut contents from both areas. Then, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) and Linear discriminant analysis Effect Size (LEfSe) were used to predict disease/pathogen related functional genes. This study revealed Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria as the predominant phyla in both studied areas. In contrast, bacterial community profiles of disturbed and undisturbed areas were slightly dissimilar. Metagenome predictions revealed that genes are related to metabolism, environmental information processing, genetic information processing, cellular processes, human diseases, and

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organismal systems. Further investigation revealed six potential biomarker genes, including chronic myeloid leukaemia in an undisturbed area, *Vibrio cholerae* infection, bladder cancer, pathogenic *Escherichia coli* infection, *Staphylococcus aureus* infection, and pertussis in disturbed areas. This study revealed that the fish gut microbiome could be used as an indicator in comparing the undisturbed and disturbed ecosystems.

Keywords: Composition, diversity, fish gut microbes, functional genes, peat swamp forests, taxa biomarkers

INTRODUCTION

Tropical peat swamp forest is one of the few biospheres considered an extreme ecosystem, usually characterised by low pH, anaerobic or low oxygen, acidic, and oligotrophic conditions (Spahni et al., 2013). There are about 882,000 hectares (ha) of remaining peat swamp forest (PSF) in Malaysia, where approximately 39% of the forests are located in Peninsular Malaysia (Posa et al., 2011). Specifically, North Selangor Peat Swamp Forest (NSPSF), covers an area of 81,304 ha and is the second-largest contiguous PSF in Peninsular Malaysia (Selangor State Forestry Department [SSFD], 2014).

Peat swamp is home to many aquatic and terrestrial macro and microorganisms (Too et al., 2018). These macro and microorganisms interact with each other in a complex and dynamic way. Microorganisms play significant roles in maintaining the equilibrium and functioning of the peat swamp ecosystem and facilitate the formation of peat, sequestration of carbon, and nutrient recycles (Chávez-Romero et al., 2016). Moreover, microbes maintain the health of the ecosystem, and host organisms residing in it (R. Zhang et al., 2020; T. Zhang et al., 2016).

A total of 198 peat swamp-associated fish species have been recorded in Malaysia. From this number, a total of 114 species from 23 families, representing about 40% of the known fish fauna in Peninsular Malaysia, were recorded from NSPSF alone (Sule et al., 2016). Fish in peat swamp is an essential part of the food web as it is entirely dependent on peat swamp habitats (Posa et al., 2011). The fish gut environment has a crucial role in maintaining metabolic homeostasis in the fish (Li et al., 2020). Many bacteria are considered desirable to the host and display certain beneficial biological activities to promote the healthy growth of the host (Banerjee et al., 2000; X. Wang et al., 2019). Gut bacteria also trigger the development of the immune system, nutrient assimilation, vitamin biosynthesis and energy metabolism, and growth (Guivier et al., 2020).

The next-generation sequencing (NGS) methods allowed us to investigate the entire complement of organisms inhabiting a particular environment. The availability of bioinformatics tools, such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) to predict the functions of 16S rRNA gene sequences, is desirable for microbial ecologists. It allows them to study the metagenomes of complex microbial communities with reasonable precision and

confidence at a high taxonomic resolution, while being able to construct robust hypotheses for further works (Langille et al., 2013; Sedlar et al., 2017). In this study, the diversity, composition, potential taxa biomarkers, and functional genes of fish gut microbes found in the extreme environment of peat swamp forests and its converted areas in North Selangor, Malaysia were explored. The fish gut microbiome was hypothesised to be used as an indicator in assessing and comparing the pristine peat swamp forest and its converted areas.

MATERIALS AND METHODS

Study Area

This study was conducted in two main areas, namely, in the undisturbed North Selangor Peat Swamp Forest (NSPSF) (undisturbed forest: UF), Malaysia and adjacent disturbed areas surrounding the NSPSF (disturbed forest: DF). The undisturbed and disturbed areas are already classified by previous studies (Sule et al., 2019). Three sampling sites in NSPSF were selected, which were in Sungai Karang Peat Swamp Forest site 1 (UF1; GPS coordinate at 3°30'00.1"N 101°12'08.7"E), Raja Musa Peat Swamp Forest (UF2; 3°29'30.7"N 101°21'51.1"E), and Sungai Karang Peat Swamp Forest site 2 (UF3; 3°41'47.9"N 101°11'03.7"E). Whereas, the sites in the adjacent disturbed area comprises of paddy field (DF1; 3°29'25.8"N 101°11'23.2"E), fire forest (DF2; 3°28'00.6"N 101°26'28.9"E), and oil palm plantation (DF3; 3°42'30.9"N 101°11'04.0"E) areas (Figure 1).

In general, for the undisturbed area, the UF1 site having a high water level and tall trees that almost covering the sampling site,

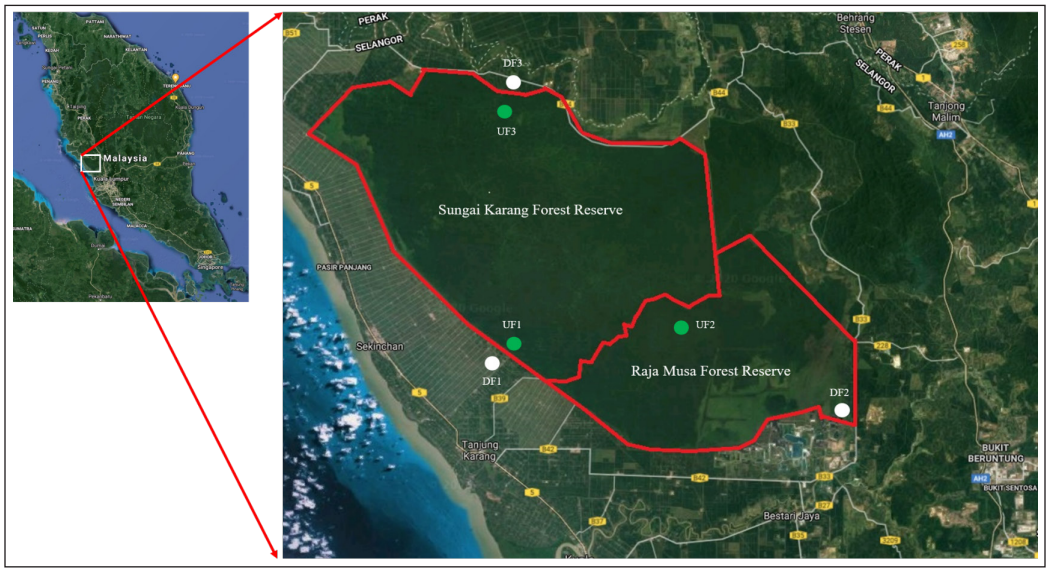


Figure 1. The map of North Selangor Peat Swamp Forest, Selangor, Malaysia. The sampling sites of UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; and UF3: Sungai Karang Peat Swamp Forest site 2 (undisturbed area; green dots), while adjacent to North Selangor Peat Swamp Forest are DF1: Paddy field; DF2: Forest fire; and DF3: Oil palm plantation (disturbed area; white dots)

sometimes with more than 1 m of depth. UF2 was the second site of the undisturbed area. The forest floor is covered by water. The water depth is averagely about 1 m in height and clearer than the UF1 site. On the other hand, the UF3 site has a high water level but is generally lower than the UF1 and UF2.

However, for the disturbed area, they are said to be anthropogenically altered. The three sites were typically artificial freshwater swamps following conversion of the peat swamp. The entire areas within the sites were majorly exposed to direct sunlight with shrubs and few trees at the bank. These sites were subjected to changes from the peat swamp forest, as evident in the peat characteristics retained in the soil and water. The DF1 site is a mixture of residential buildings and other human activities, such as the main road. The DF2 site shows few signs of irrigated canals and experienced forest fire several years ago, as young trees and plantations can be observed. On the other hand, the DF3 site was covered by oil palm plantations.

Fish Sampling

The fish samples were randomly collected in December 2018 using a combination of gill, cast, and scoop nets in each sampling sites. The weight and length of each fish were measured using an electronic weighing balance and a vernier calliper, respectively. The collected fishes were then placed in a 30 cm long × 60 cm wide × 50 cm high plastic container, and they were euthanised with an overdose of 250 mg/L of MS-222 (Sigma-Aldrich, United States of America). The abdomen was opened at the ventral midline by inserting a fine scalpel blade into the anus of the fish. At the same time, the incision was extended anteriorly, and the gastrointestinal contents (GI) were removed under aseptic conditions. The GI samples were then placed in sterile 50 mL falcon tubes containing RNALater solution (Thermo Fisher Scientific, United States of America) stored at -20ºC. The fish were sampled, handled, and sacrificed according to the methods approved by Institutional Animal Care and Use Committee, Universiti Putra Malaysia. All methods were carried under relevant guidelines and regulations. The permit to access and collect samples was obtained from Selangor State Forestry Department, Selangor [Ref. no: P.H.D. 600-5/2/6(19)], while none of the sampled fish species used in this study was considered endangered and protected by the government of Malaysia.

DNA Extraction, PCR, and Sequencing Library Preparation

FavorPrep™ Stool DNA Isolation Mini Kit (Favorgen, Taiwan) was used for DNA extraction of fish gut contents. The quantity and purity of the extracted DNA were then measured and checked using NanoDrop™ 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, United States of America) and 1% agarose gel electrophoresis. For further analyses, the concentration of DNA was determined at > 40 µg; meanwhile, the purity of the excellent quality DNA will have an A260/280 ratio of 1.8 – 2.0. The

extracted DNA of the fish gut contents was stored in a freezer at -80°C until further analyses.

The PCR mixture and the thermal cycling were carried out in duplicates according to Q. Wang et al. (2017) for each extracted DNA. The reactions in a final volume of 25 µL were prepared to contain the following 2.5 µL of 5 ng/µL DNA extract, 5 µL of 0.2 µM 16S (V3-V4) forward primer of 5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG 3' and 0.2 µM of 5 µL reverse primer of 5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C 3' and 12.5 µL of master mix (DreamTaq Green PCR Master Mix (2×), Thermo Fisher Scientific, United States of America) containing 1.0 µL of 0.2 µM *Taq* polymerase, 2.5 µL of 1 µM buffer, 0.5 µL of 200 µM dNTP, 1.0 µL of 2.0 mM $MgCl_2$, and 7.5 µL of sterilised distilled water. The PCR reaction was carried out in Mastercycler® Nexus Gradient (Eppendorf®, Germany) with an initial denaturation at 95ºC for 3 min, 30 cycles of denaturation at 95ºC for 30 sec, annealing at 55ºC for 30 sec, elongation at 72ºC for 30 sec, extension at 72ºC for 5 min and finally, hold at 10ºC. In addition, gel electrophoresis was carried out on DNA samples.

A total of six DNA samples (three replicates of pooled DNA samples × two undisturbed and disturbed areas) were sent for DNA sequencing on the Illumina Miseq (United States of America) sequencing platform in Novogene Biological Information Technology Co. (China) through Apical Scientific Sdn. Bhd., Malaysia, which results in 250 bp paired-end reads.

Analysis of 16S rRNA Amplicon Sequencing

The raw MiSeq sequencing results in fastq. format was pre-processed and analysed using Quantitative Insights into Microbial Ecology (QIIME ver. 1.9.1 64-bit) pipeline (Caporaso et al., 2010). All sequences were trimmed to remove the primer and barcode sequences using fast length adjustment of short reads (FLASH) (v2.0) (Magoč & Salzberg, 2011) and merged through paired-end read merger (PEAR) (J. Zhang et al., 2014). The reads in fastq. file were subjected to quality filtering at Phred Quality Score of $q = 20$ and $p = 70$ using fastq_quality_filter under fastx_toolkit (http://hannonlab.cshl.edu/fastx toolkit/). Chimeric sequences were screened using UCHIME, against the RDP_GOLD (v9) database and were removed from the downstream processing (Haas et al., 2011). The short reads may lead to an unspecific match that could interfere with accuracy. Therefore, to increase the accuracy of the calling reads shorter than 100 bp or longer than 600 bp were removed along with the low-quality bases ($Q < 33$). The fastq was converted to fasta file by fastq_to_fasta in FastX-toolkit in QIIME. Operational taxonomic unit (OTU) was selected at least 97% similarity threshold using the pick_otus.py script with the usearch_ref method against the Greengenes database through closed references OTU picking. OTU table was

constructed containing the abundance and taxonomic assignments of all OTUs.

Diversity Analysis and Top Percentage Contribution of Taxa

Alpha diversity indexes were calculated to explain the species richness and diversity in each sample. At the same time, rarefactions curves were plotted to determine the adequacy of sequencing depth (Udayangani et al., 2017). A total of 674,795 (≥ 500 bp) clean reads were used to get the best quality in calculating the observed species, Chao1, seChao1, ACE, se.ACE, Shannon, Simpson, InvSimpson, and Fisher.

Beta diversity using Bray-Curtis distance was also determined (Bray & Curtis, 1957). The permutation-based multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) was used to test the taxonomic dispersion homogeneity across the areas with 2D estimated stress. The computation of Bray-Curtis, PERMANOVA, and ANOSIM was conducted using PAleontological STatistics (PAST) software (v3.11) (Hammer et al., 2001). The OTU table of the disturbed and undisturbed samples was used to generate non-metric multidimensional scaling (NMDS) in PAST3. Principal component analysis (PCA) was used to visualise similarities or dissimilarities of data based on phylogenetic or distance metrics (Jonsson et al., 2016).

Similarity percentage (SIMPER) was carried out using PAST3 software (v3.11) to determine the top percentage contribution of bacterial taxa from fish gut contents at family and genus levels in disturbed and undisturbed areas. The SIMPER results were visualised in the form of heatmaps, and extended error bars using MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/faces/docs/Contact.xhtml), Sparse Correlations for Compositional data (SparCC) network of OTU abundance at the class level of the fish gut contents in disturbed and undisturbed areas were constructed.

Predictions of Health-Related Functional Genes

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) in the Galaxy server was used to impute the prevalence of genes encoding selected bacterial pathogen/diseases, antibiotic resistance genes, degradation, and other toxic related molecules against Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database. Similarly, the presence of specific metabolic and digestive genes whose products are likely to contribute to the fish survival and well-being was determined using the Linear discriminant analysis Effect Size (LEfSe), which measured both biological relevance and statistical significance (Segata et al., 2011). For this analysis, the alpha parameter significance threshold for the Kruskal-Wallis among class was set to 0.01, and the logarithmic linear discriminant analysis (LDA) score cut-off was set to 2.0. This analysis was performed on the Galaxy server.

Statistical Analysis and Data Availability

Shapiro-Wilk's test was conducted to test for normality and homoscedasticity (Levene's test). Similarly, statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS) version 26.0 (SPSS®, United States of America), and statistical significance was determined at $p < 0.05$. The SPSS v26.0 was also used to carry out one-way ANOVA with Tukey's post-hoc test ($p < 0.05$ was considered significant) to compare the differences of alpha diversity indexes among the three disturbed and undisturbed areas. Similarly, ANOVA with Tukey's post-hoc test ($p < 0.05$ was considered significant) was carried out to determine the level of taxa (phylum, class, order, family, and genus) of statistically significant difference among the samples. All the fastq. files for 16S rRNA gene sequence data were also submitted to *National Center for Biotechnology Information* (NCBI) Sequence Read Archive (SRA) database, under the following accession numbers SAMN13909365 (DF1), SAMN13909366 (DF2), SAMN13909367 (DF3), SAMN13909368 (UF1), SAMN13909369 (UF2), and SAMN13909370 (UF3).

RESULTS

Fish Samples

This study collected a total of nine fish species. UF2 site recorded the highest fish samples with 12, while the lowest was in UF3 and DF1 sites with five fish samples. Details of the fish species, number, length and weight, are provided in Appendix 1.

Rarefaction Curves of Bacterial Communities in Undisturbed and Disturbed Areas

The rarefaction and associated diversity analysis showed some variation in bacterial diversity and taxon richness of 16S rRNA genes between undisturbed and disturbed areas. The number of new OTUs found in undisturbed and disturbed areas increased as the number of sequences increased (Figure 2). The plot was rarefied at a minimum library size (850 sequence reads). All samples reached a plateau which indicated that the maximum number of OTU had been identified.

Alpha Diversity of Bacterial Communities in Fish Gut Contents

The 16S rRNA gene sequences of bacterial communities in fish gut contents from undisturbed and disturbed areas were analysed for alpha diversity. In general, species richness was higher in the undisturbed area, in which Observed OTU, Chao1, se.Chao1, ACE, se.ACE, and Fisher had greater values than disturbed areas. On the other hand, diversity indexes such as Shannon, Simpson, and InvSimpson indexes were lower in the undisturbed area (Table 1). However, there was no significant difference ($p > 0.05$) in bacterial diversity indexes and richness among the samples.

Beta Diversity of Bacterial Community among Sites

Bray-Curtis similarity scores were inferred from the taxonomic data generated by QIIME in PAST v3.11 and consequently

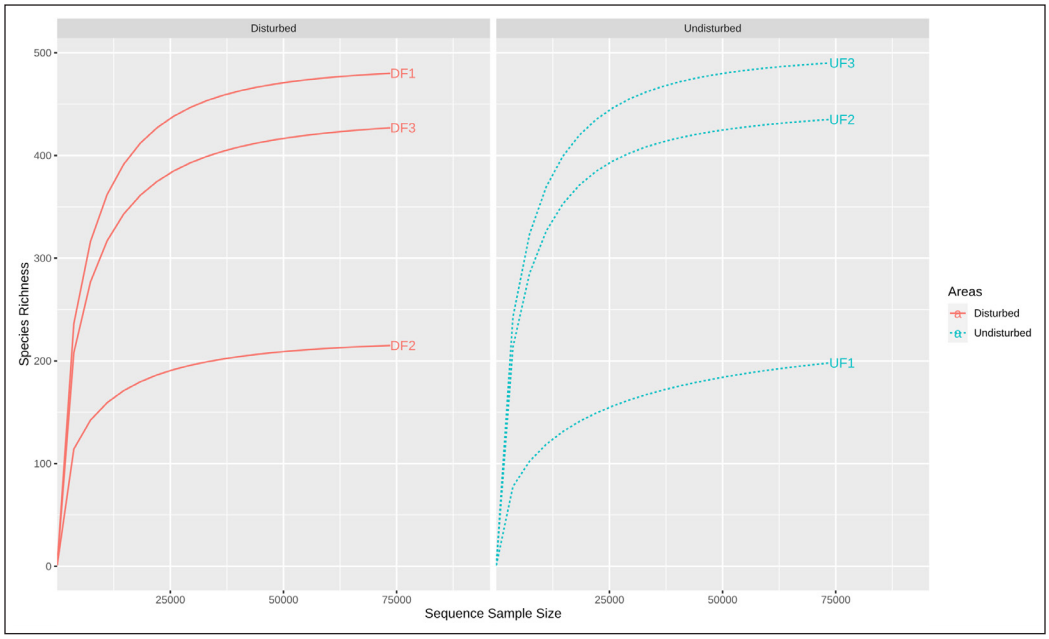


Figure 2. The rarefaction curves show the increase in bacterial operational taxonomic unit's (OTU) and the number of randomly sampled sequences of the bacteria in fish gut contents from undisturbed and disturbed areas. The OTU's detected per sample as a function of the sequencing effort. The plot was rarefied at minimum library size (850 sequence reads)
Note. DF1: Paddy field; DF2: Forest fire; DF3: Oil palm plantation; UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; UF3: Sungai Karang Peat Swamp Forest site 2

reduced to a two-dimensional space using NMDS to estimate the structural similarity between samples. Although they were slightly separated based on samples between disturbed and undisturbed areas (Figure 3a), it was statistically insignificant (p > 0.05) with a stress value of 0.1796. Figure 3b shows the number of commonly shared (core) and unique OTUs in disturbed and undisturbed samples.

Bacterial Community Structures in Fish Gut Contents

The structure of the bacterial community, particularly at phylum, class, order, family, and genus levels, are described in Appendix 2. There were about 19 - 25 phyla detected across the samples. DF2 site recorded the lowest, while UF3 site showed the highest number of phyla. A similar pattern was obtained at the genera level, having 165 and 239 genera in DF2 and UF3 areas, respectively. There is no significant difference (p > 0.05) in fish gut bacterial community structures between undisturbed and disturbed areas across taxa (phylum, class, order, family, and genus) (Appendix 3).

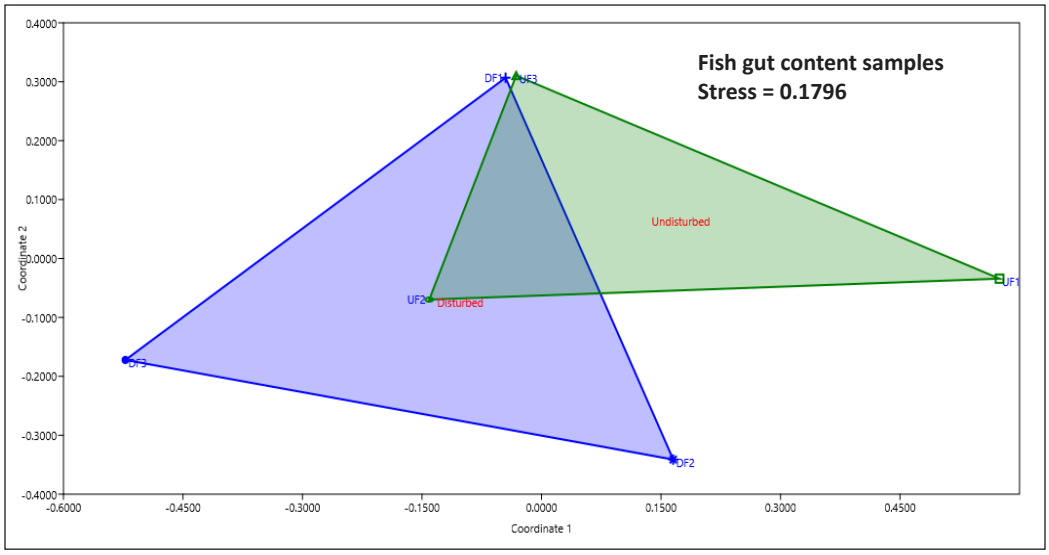
Composition of Bacterial Communities in Fish Gut Contents

A total of six samples of 16S rRNA gene libraries were generated, representing the fish gut contents from the three undisturbed

Table 1
Alpha diversity metrics of bacterial communities in fish gut contents collected from undisturbed and disturbed areas

Site	Observed OTU	Chao1	se.Chao1	ACE	se.ACE	Shannon	Simpson	InvSimpson	Fisher
DF1	1380	1596.47	30.19	1662.06	19.41	3.41	0.89	9.64	246.99
DF2	765	822.50	14.27	825.37	13.14	3.17	0.88	8.77	121.53
DF3	1554	1959.61	47.87	1995.24	22.16	2.43	0.59	2.47	285.51
UF1	1265	1599.94	42.53	1680.64	21.18	2.07	0.54	2.20	2.22
UF2	1596	1853.38	34.80	1863.69	19.94	2.77	0.63	2.74	294.99
UF3	1509	1846.59	41.96	1890.60	21.40	3.54	0.90	10.63	275.43
DF	1233.00 ± 414.53	1459.53 ± 580.79	30.78 ± 16.80	1494.2 3± 602.72	18.24 ± 4.62	3.01 ± 0.51	0.79 ± 0.17	6.97 ± 3.91	218.01 ± 85.74
UF	1456.67 ± 171.59	1766.64 ± 144.40	39.76 ± 4.31	1811.65 ± 114.25	20.85 ± 0.78	2.80 ± 0.74	0.70 ± 0.19	5.19 ± 4.72	264.22 ± 37.66

Note. DF1: Paddy field; DF2: Forest fire; DF3: Oil palm plantation; UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; UF3: Sungai Karang Peat Swamp Forest site 2; DF: Disturbed forest area; UF: Undisturbed forest area



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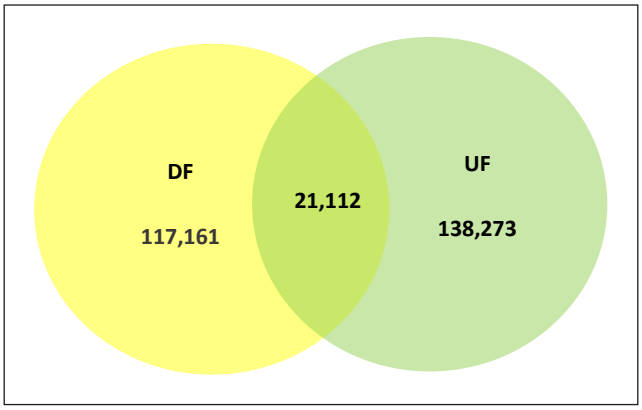


(b)

Figure 3. a) Comparison of NMDS ordination of bacterial communities in fish gut contents collected from undisturbed and disturbed areas with ANOSIM at R = 0.2222. b) Venn diagram summarising the number of core and unique OTUs in disturbed and undisturbed samples

Note. DF1: Paddy field; DF2: Forest fire; DF3: Oil palm plantation; UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; UF3: Sungai Karang Peat Swamp Forest site 2; DF: Disturbed forest area; UF: Undisturbed forest area

and disturbed areas. The Proteobacteria (36.69%) was the predominant phylum in undisturbed area samples and across all the samples. However, Firmicutes (26.95%) and Bacteroides (4.36%) were the members of the phyla that were higher in disturbed area samples (Figure 4a).

Enterobacteriaceae (42.64%) is the most common family across all samples and is higher in an undisturbed area at the family level. In comparison, additional families present include Ruminococcaceae (10.62%) and Erysipelotrichaceae (9.27%). Whereas, Clostridiaceae (14.63%), Rikenellaceae

(10.94%), and Fusobacteriaceae (7.02%) were also present in disturbed area samples (Figure 4b).

The comparison at the genus level revealed that *Clostridium*, *Providencia*, and *Cetobacterium* representing 28.73%, 22.13%, and 19.14%, respectively were the most dominant genera, particularly in disturbed area samples. On the other hand, the top members recorded in undisturbed area samples include *Lactococcus* (16.36%), *Bacteroides* (12.07%), *Desulfovibrio* (11.30%), and *Candidatus* Arthromitus (6.97%) (Figure 4c).

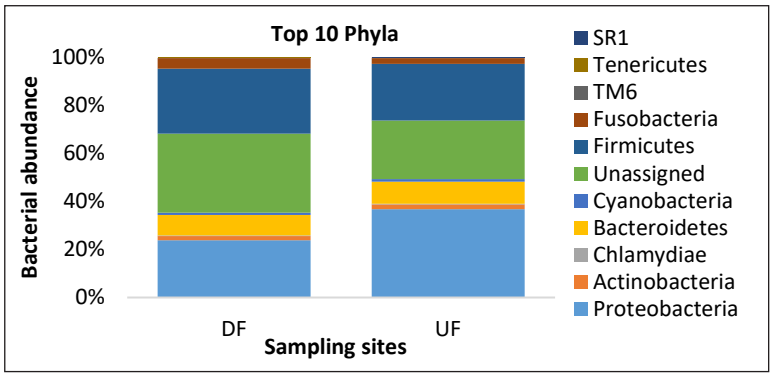
Percentage Contribution of Bacterial Taxa

The percentage contribution of bacterial taxa was determined at the family level, and overall average dissimilarities showed 46.08 (Appendix 4). The results revealed that Enterobacteriaceae (77.90%) and Streptococcaceae (6.70%) recorded a higher percentage contribution in an undisturbed area, particularly in the UF1. On the other hand, the disturbed area recorded a higher percentage contribution of families Clostridiaceae (19.20%) and Peptostreptococcaceae (10.30%) in the DF2 area, while Fusobacteriaceae (6.15%) in the DF3 area. Comparison of disturbed and undisturbed sample areas showed Clostridiaceae, Fusobacteriaceae, Erysipelotrichaceae, and Peptostreptococcaceae were family members with a higher percentage contribution in the disturbed area. Conversely, Enterobacteriaceae, Ruminococcaceae, Streptococcaceae, and Desulfovibrionaceae families were more prevalent in undisturbed areas (Figure 5a).

Figure 5b shows the top 15 contributors at the genus level. The high percentage contributors of taxa in undisturbed and disturbed areas were *Providencia* (15.10%) and *Weissella* (0.98%), respectively. Whereas, *Desulfovibrio* (5.09%) and *Methylosium* (1.44%) were recorded in the UF1 area. In contrast, the disturbed area samples showed *Lactococcus* (6.57%), *Enterobacter* (1.61%), *Turicibacter* (1.12%), *Pseudomonas* (0.64%), and *Epulopiscium* (0.39%) were mainly higher in the DF1 area. At the same time, *Bacteroides* and *Candidatus* Arthromitus have the highest percentage contribution in the DF3 area, represented by 5.33% and 2.67%, respectively. Thus, the results indicated relatively higher *Providencia*, *Clostridium*, *Cetobacterium*, *Desulfovibrio*, and *Weissella* in undisturbed than in disturbed area samples. However, the genera of *Lactococcus*, *Enterobacter*, *Turicibacter*, *Bacteroides*, *Candidatus* Arthromitus, and *Methylosium* were predominant in the disturbed area.

Co-occurrence Networks between Different Fish Gut Bacterial Classes

The SparCC was used to elucidate the networks of interaction among the classes of fish gut microbiota. The bacterial abundance in disturbed and undisturbed areas showed significant associations among the taxonomic clades in a single giant cluster with one main taxa, Deferribacteres in the



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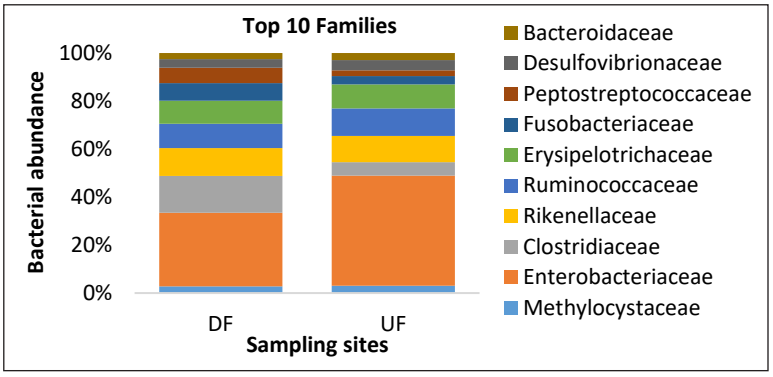

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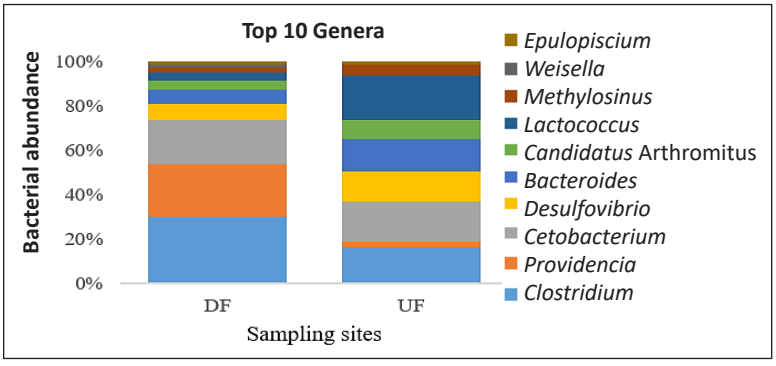
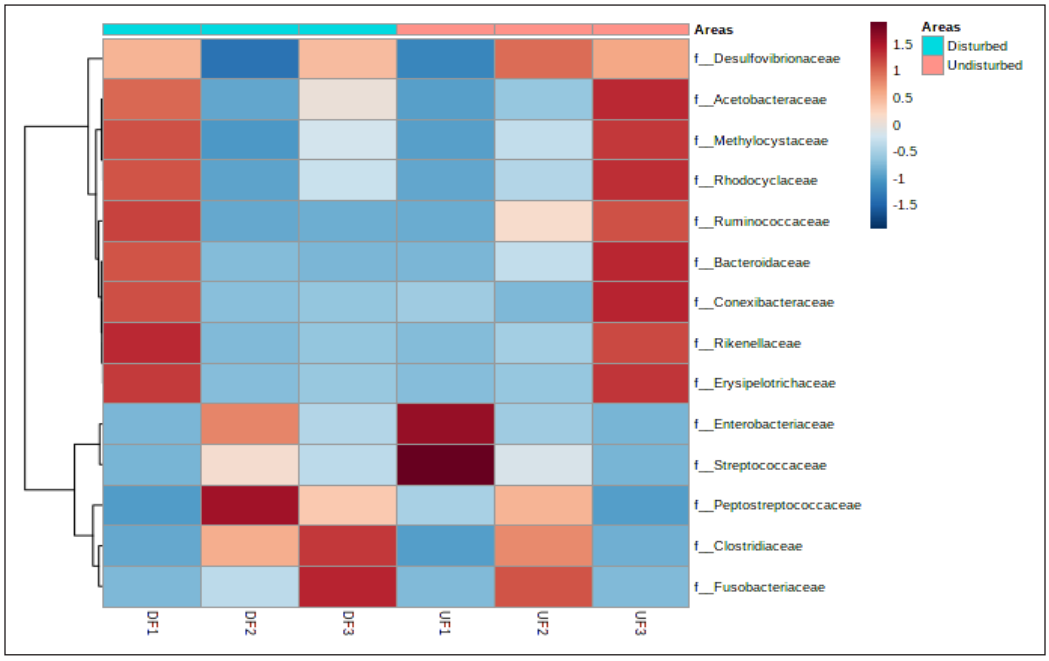


(c)

Figure 4. Comparison of relative abundances of top 10 bacterial communities in gastrointestinal contents collected from disturbed and undisturbed areas: (a) Phyla; (b) Family; and (c) genus level
Note. DF: Disturbed forest area; UF: Undisturbed forest area

centre of the cluster, having positive or negative correlations to the other four sub-clusters (Figure 6). The class Deferribacteres linked with the first sub-cluster through *Chlamydia* and second sub-cluster via Erysipelotrichia, while Bacteroidia bonded the third sub-cluster by class SJA_4. All the networks depicted either positive or



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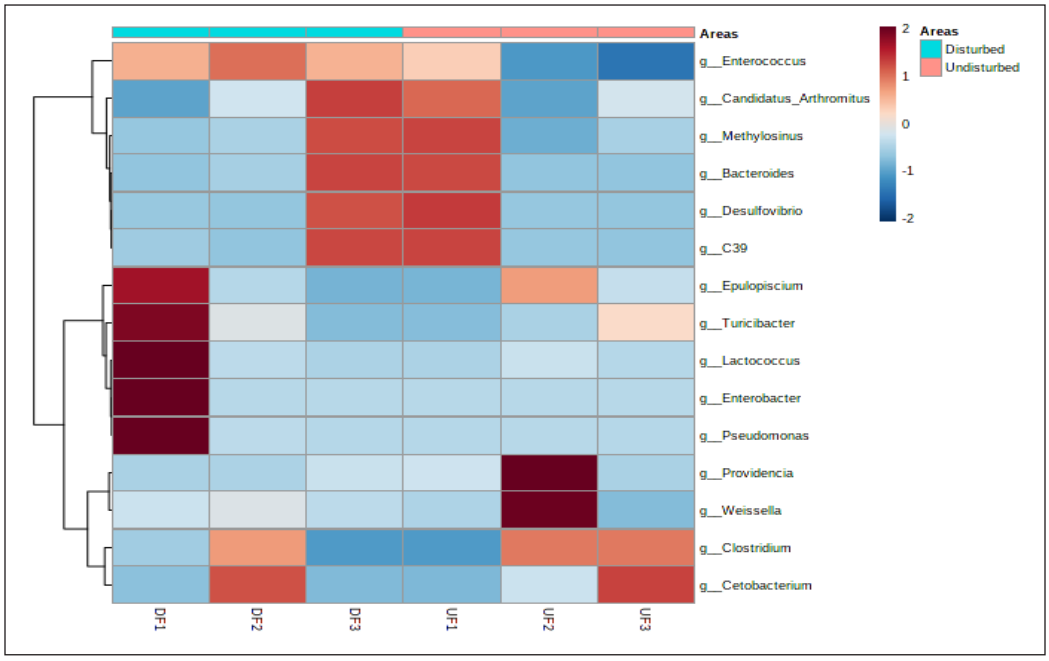


(b)

Figure 5. Heatmaps of top 15 high percentage contribution of bacterial abundance at a) family and b) genus level across samples of fish gut contents collected from disturbed and undisturbed areas. ANOSIM showed no significant difference ($p > 0.05$) in fish gut contents collected from disturbed and undisturbed areas
Note. DF1: Paddy field; DF2: Forest fire; DF3: Oil palm plantation; UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; UF3: Sungai Karang Peat Swamp Forest site 2

negative associations. The first sub-cluster, Acidobacteria, showed a strong and positive association between Nostocophycideae and Ocillatoriophycideae, prevalent in the disturbed area. The Flavobacteriia recorded higher abundance in the undisturbed area and correlated positively with Acidobacteria. However, *Chlamydia* showed a negative and strong correlation with the three classes.

The second sub-cluster linked to Deferribacteres was prevalent in the disturbed area with a positive and strong association with Betaproteobacteria and Bacteroidia. On the other hand, Deltaproteobacteria were higher in the undisturbed area, negatively and strongly correlated with Fusobacteria. Deferribacteres displayed positive associations with Erysipelotrichi, *Chlamydia*, Betaproteobacteria, ABS_6, and Bacteroidia, forming the third sub-cluster. Finally, the fourth sub-cluster revealed Thermoplasmata exclusively found in the disturbed area, having negative and strong correlations with Methanobacteria and SJA_4. In contrast, class SJA_4 was correlated positively with Methanobacteria and Bacteroidia.

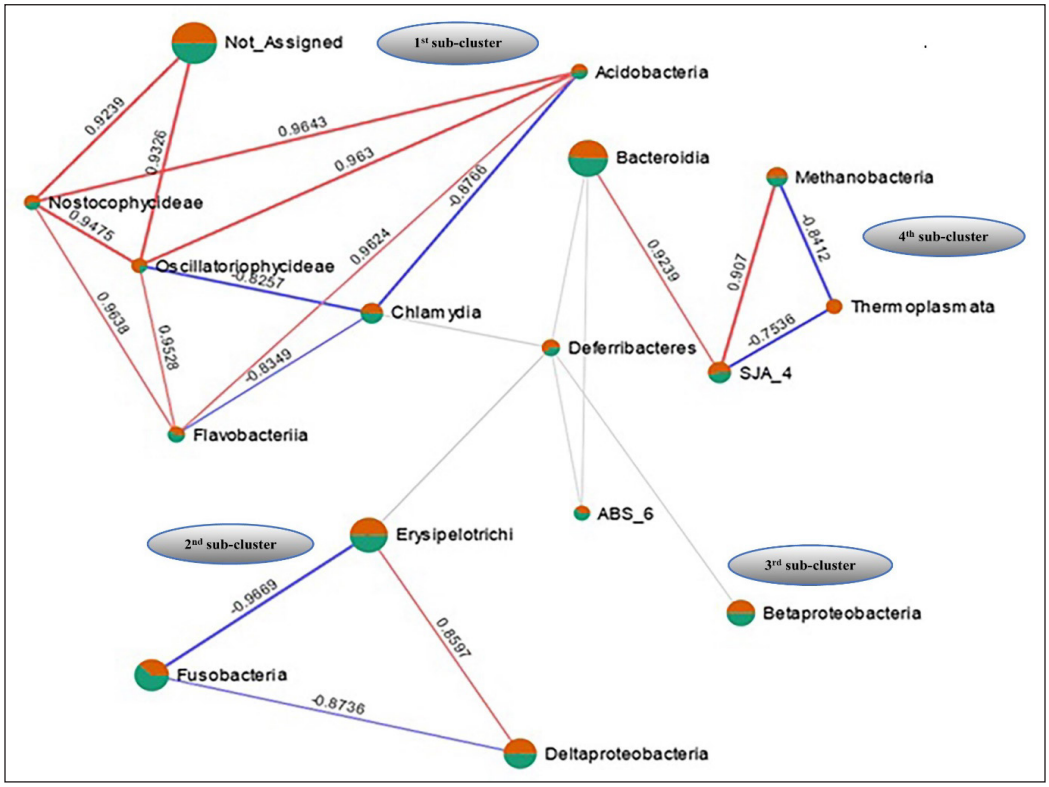
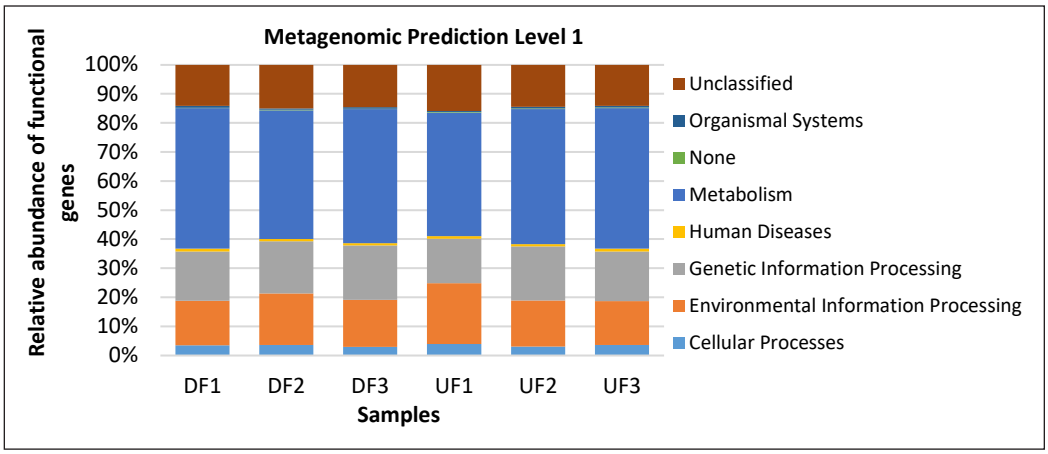


Figure 6. SparCC network of the mean OTU abundance at a class level from fish gut contents collected from disturbed and undisturbed areas at 200 permutations. The significant correlations exhibited between the classes of bacteria at p value = 0.05 and r = 0.7 thresholds. The taxonomic clades are represented in blue circles. The fish gut samples collected from the disturbed area are represented by orange colour. In contrast, fish gut contents collected from the undisturbed area were labelled green. Additionally, the thicker line indicates a more positive or negative association between or among taxa. In contrast, the thinner lines showed a lesser association

Functional and Biomarker Genes Associated with Fish Health

PICRUSt analysis predicted eight primary functional genes in KEGG pathways (KEGG level 1). Metabolism was comparatively higher in undisturbed area samples. Others include environmental information processing, genetic information processing, unclassified, cellular processes, human diseases, organismal systems, and none recorded the least functional genes across all samples (Figure 7a).

Health-related functional genes from PICRUSt prediction were sorted, and LEfSe was used to find health-related biomarkers. The abundance of the biomarker genes in fish gut samples from undisturbed and disturbed areas was compared. LEfSe identified six biomarker genes associated with diseases, antibiotics, and toxins in the samples. The biomarkers include chronic myeloid leukaemia, *Vibrio cholerae* infection, bladder cancer, pathogenic *Escherichia coli* infection, *Staphylococcus aureus* infection,



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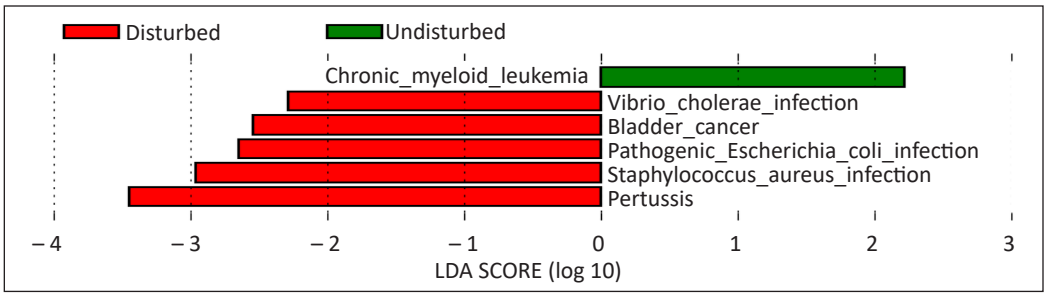

(b)

Figure 7. (a) Relative abundance of predicted KEGG Orthologs functional profiles (KEGG level 1) of fish gut bacterial community was collected from disturbed and undisturbed areas. The results were based on log2 (+1 transformed values). In addition, the functional prediction between groups was tested using bootstrap Mann-Witney U-test with cut off at $p < 0.05$; (b) LEfSe of pathogen or disease-related functional biomarker genes at KEGG level 3 in disturbed and undisturbed samples of fish gut contents

Note. DF1: Paddy field; DF2: Forest fire; DF3: Oil palm plantation; UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; UF3: Sungai Karang Peat Swamp Forest site 2

and pertussis. Except for chronic myeloid leukaemia, the rest were mainly associated with disturbed area samples. Interestingly, pertussis and *S. aureus* infection were over-represented in the disturbed area (Figure 7b).

DISCUSSION

In this study, following our fish sampling activities, we could not find a uniform fish species representing a fair comparison between the undisturbed and disturbed areas and within the undisturbed and disturbed areas themselves. However, all fish used in this study were primarily omnivorous, which feed on various food of plant and animal (insects) origin. Thus, it will rule out the bias in the context of fish feeding behaviour. Moreover, samples from each site were pooled to represent the fish gut microbiome data from each area.

Alpha diversity measures of the bacterial community in the fish gut contents were rich and diverse. In the peat forest, relatively higher bacterial diversity in undisturbed soil was also reported (Sun et al., 2014). By implication, the comparative low bacterial diversity in the disturbed area might be affected by effluents produced by anthropogenic activity, which appeared higher in the disturbed area. The rarefaction curve began to reach a plateau pattern, suggesting decreasing frequency of discovery of new taxa with increasing sequencing depth. The number of species becomes saturated, regardless of increased sampling frequency. The rarefaction curves indicate that the sampling sufficiently captures the species in the study area. Unique taxa recorded in both disturbed and undisturbed areas indicated that the bacterial communities are most likely reflect by the environmental condition surrounding the host, which in this study is the anthropogenic factors (Nayak, 2010; Nolorbe-Payahua et al., 2020).

The tropical peat swamp forests are nutrient and oxygen-deficient (Aw et al., 2016), displaying unique systems with core microbial assemblages, such as Proteobacteria, Bacteroides, Actinobacteria, Cyanobacteria, Chlamydiae, and some other phyla. Fish intestines particularly harbour large and diverse populations of bacteria (Givens et al., 2015). Similar bacterial phyla and families were also predominant in NSPSF (Too et al., 2018). Proteobacteria can withstand polluted and extreme environments as in the peat swamp forests (Ward et al., 2009). Members of the proteobacterial methanotrophs, such as the genera *Methylomonas*, *Methylobacter*, *Methylococcus* (type I), *Methylosinus*, and *Methylocystis* (type II), oxidise atmospheric and subsurface methane before it can be released into the atmosphere (Nguyen et al., 2018). This process explains the prevalence of the phylum Proteobacteria in the fish gut from undisturbed area samples.

This study found a change in bacterial diversity in fish microflora inhabiting a disturbed forest compared to an undisturbed forest area geographically distant from any possible source of human activities and pollutants. Enterobacteriaceae appeared to be dominant in undisturbed than disturbed

area samples. Other prevalent members in the disturbed area included Clostridiaceae, Fusobacteriaceae, Erysipelotrichaceae, and Methylocystaceae. The results showed differences between undisturbed and disturbed areas on the fish gut microbiome composition, confirming the previous finding that the surrounding habitat influences the microbial flora in the fish intestinal tract (Sule et al., 2019). However, the composition of the fish gut bacterial community varied depending on habitat (Yukgehnaish et al., 2020). Enterobacteriaceae is a sizeable Gram-negative group with more than 50 genera and over 200 species (Don et al., 2005). Members of this family are frequently found in the normal microbiota of the fish gut (Egerton et al., 2018; Oliveira et al., 2017). Therefore, it might be among the important resident microbiota having a symbiotic relation to its host (Yukgehnaish et al., 2020). However, the family members vary, from beneficial or harmless to several pathogenic members such as *Salmonella*, *Escherichia*, *Yersinia*, *Klebsiella*, *Shigella*, *Serratia*, and *Proteus* (Don et al., 2005). Moreover, the higher abundance of Enterobacteriaceae in the fish gut sample of the undisturbed area could be due to its important role as a core microbiota in the fish gut. In contrast, Enterobacteriaceae in the disturbed area has low abundance compared to the undisturbed area. At the same time, Clostridiaceae and Fusobacteriaceae were found higher abundance in disturbed compared to the undisturbed area. Hence, the impacts of anthropogenic activities and the by-product carried out in that area can alter the core microbiome of fish species. This finding agrees with the other previous studies that were suggesting that environmental waste causes changes in the gut microflora of aquatic species. The fish species exposed to anthropic contaminants within their ecosystem lead to changes in microbial composition (Evariste et al., 2019; Giang et al., 2018). In the present study, we found bacterial groups in fish guts that could represent potential biomarkers of environmental contamination by anthropogenic activities. Furthermore, genus *Clostridium* was observed more abundant in disturbed compared to undisturbed areas. *Clostridium* were considered one of the markers for persistent faecal contamination in stream water (Mushi, 2018). Hence, the presence of *Clostridium* in fish gut suggests that the fish's environment was disturbed by anthropogenic influences.

Several functional genes related to health consequences in fish were deduced from the predicted PICRUSt results. The health-related genes were sorted, and differentially abundant features in undisturbed and disturbed areas were analysed using LEfSe. Bladder cancer might be associated with oxidative DNA damage, which plays an important role in the pathogenesis of some human diseases, including cancer (Szymańska & Długosz, 2017). Fusobacteriacea and *Acinetobacter* were among the taxa with the maximum percentage contribution in the undisturbed fish gut samples. Moreover, *Fusobacterium nucleatum* and some members of the Ruminococcaceae family (Bučević et al., 2018) and *Acinetobacter* (Mai et al., 2019)

were detected in patients with bladder cancer. Thus, genera *Fusobacterium* and *Acinetobacter* could be a potential protumorigenic pathogen.

Other detected functions are chronic myeloid leukaemia and *Staphylococcus aureus* infection. Similarly, chronic myeloid leukaemia is associated with increased and unregulated growth of myeloid cells in the bone marrow and blood (Nowell, 2007). On the other hand, the pathogenic *Escherichia coli* infection is responsible for enteropathogenic and enterohaemorrhagic *E. coli*. It is causing gastroenteritis and bloody diarrhoea in infants, posing a major threat to human health, especially in the developing world (Humphreys et al., 2016). *Staphylococcus aureus* infection is linked with humans and animals' infection. The presence of staphylococci in fish indicating contamination has occurred. The situation is worrisome as it is a harmful human pathogen causing nosocomial infections. Fish consumption contaminated with *S. aureus* could lead to food poisoning (Hammad et al., 2012). *Vibrio cholerae* pathogenic cycle is connected to infective stages of *V. cholerae*, a cosmopolitan bacterium that inhabits a vast range of environments and is associated with many aquatic organisms. *Vibrio cholerae* possesses a diverse metabolism characterised by rapid inter-regulation of energy-producing pathways to adapt between environments (Bueno et al., 2020). The bacterium is found in water contaminated with human faeces/sewage. It is considered a significant source of pathogenic bacteria responsible for cholerae in humans (Pandey & Mishra, 2020). Lastly, pertussis is associated with a respiratory infectious disease caused by a pathogen, *Bordetella pertussis*. The disease is characterized by paroxysmal cough, inspiratory wheezing, and vomiting (Beier & Gross, 2006).

CONCLUSION

The results revealed a high diversity of microbes in fish gut contents collected from undisturbed and disturbed areas. However, the species richness was relatively lower in disturbed areas. The partial overlap between the undisturbed and disturbed area samples in NMDS analysis suggests high similarity in the composition of the fish gut bacterial community. There could be a possible movement of fishes from disturbed to undisturbed areas, or *vice versa* during high water levels in raining season. Moreover, omnivorous feeding behaviours of all the collected fishes also might be the factor. Rarefaction analysis indicates sufficient sequencing depth. Overall, fish gut bacterial communities in the disturbed area showed a lower total absolute count than the undisturbed area. This study revealed that the fish gut microbiome could be used as an indicator in accessing and comparing the healthy and disturbed ecosystems.

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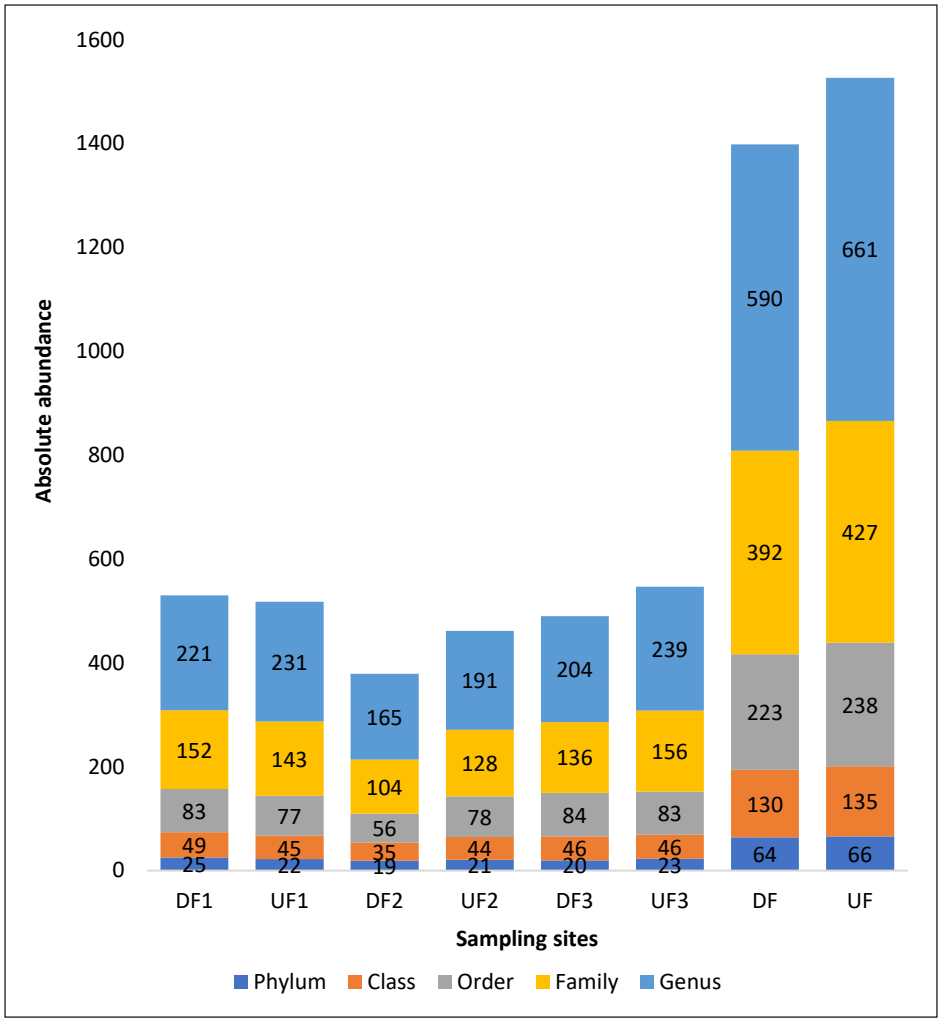
APPENDICES

Appendix 1
The collected fish species in this study

Sites	Fish species	Length (cm)	Weight (g)
UF1	*Pristolepis fasciata*	4.5	4.3
	Anabas testudineus	3.5	0.5
	Anabas testudineus	4.4	4.3
	Anabas testudineus	2.6	0.3
	Trichopsis vittata	6.7	2.0
	Pristolepis fasciata	4.0	0.5
UF2	*Mystus singaringan*	11.5	9.0
	Hampala macrolepidota	8.0	5.0
	Trichopsis vittata	11.5	10.0
	Rasbora dusonensis	12.0	16.0
	Trichopsis vittata	7.0	2.0
	Pristolepis fasciata	5.0	2.0
	Rasbora dusonensis	10.5	9.0
	Trichopsis vittata	6.2	2.0
	Hemibagrus capitulum	6.0	1.0
	Pristolepis fasciata	6.0	5.0
	Trichopsis vittata	4.5	1.0
	Trichopsis vittata	6.7	2.0
UF3	*Helostoma temmincki*	4.5	4.3
	Pristolepis fasciata	5.6	4.0
	Anabas testudineus	5.8	4.0
	Anabas testudineus	5.7	3.0
	Anabas testudineus	6.3	5.0
DF1	*Pristolepis fasciata*	7.0	5.0
	Trichopodus trichopterus	7.0	4.0
	Trichopodus trichopterus	6.0	3.0
	Trichopodus trichopterus	5.5	2.0
	Pristolepis fasciata	5.0	4.0
DF2	*Pristolepis fasciata*	6.5	4.0
	Anabas testudineus	4.5	1.0
	Pristolepis fasciata	6.7	6.0
	Anabas testudineus	5.0	7.5
	Pristolepis fasciata	6.5	5.0
	Anabas testudineus	5.0	7.0
	Anabas testudineus	5.5	8.0
	Anabas testudineus	5.0	7.0
	Anabas testudineus	6.5	5.0
DF3	*Trichopodus trichopterus*	2.5	0.2
	Trichopodus trichopterus	3.4	0.4
	Trichopodus trichopterus	2.1	0.2
	Trichopodus trichopterus	2.4	0.2
	Trichopodus trichopterus	2.3	0.2
	Trichopodus trichopterus	3.5	1.0

Note. UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; UF3: Sungai Karang Peat Swamp Forest site 2; DF1: Paddy field; DF2: Forest fire; DF3: Oil palm plantation



Appendix 2. Bacterial community structures of fish gut contents collected from disturbed and undisturbed area across phylum, class, order, family, and genus displayed in the absolute abundance
Note. DF1: Paddy field; DF2: Forest fire; DF3: Oil palm plantation; UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; UF3: Sungai Karang Peat Swamp Forest site 2; DF: Disturbed forest area; UF: Undisturbed forest area

Appendix 3
One way-ANOSIM of fish gut bacterial community structures between undisturbed and disturbed area across taxa (phylum, class, order, family and genus)

Permutation N:	9999
Mean rank within:	8.5
Mean rank between:	7.667
R:	-0.1111
p (same):	0.9038

Note. N: Size of the population; R: Size of each sampling group; *p*: *p*-value

Appendix 4

Top 15 % contribution of family level in gastrointestinal contents collected from disturbed and undisturbed areas

Taxon	Av. dissim	Contrib. %	Percentage contribution of taxa					
			DF1	DF2	DF3	UF1	UF2	UF3
Unassigned	19.53	27.03	1.33	2.89	77.00	2.45	70.10	1.22
f__Enterobacteriaceae	17.54	24.28	2.83	52.00	2.76	77.90	2.24	2.69
f__Rikenellaceae	5.913	8.184	23.10	0.00	0.22	0.07	0.54	20.60
f__Ruminococcaceae	5.266	7.288	20.10	0.30	0.15	0.63	2.39	19.10
f__Erysipelotrichaceae	5.075	7.025	19.00	0.04	0.22	0.03	0.23	19.10
f__Clostridiaceae	3.198	4.427	2.89	19.20	5.53	1.93	5.10	3.48
f__Peptostreptococcaceae	1.832	2.535	0.46	10.30	1.12	2.38	1.44	0.52
f__Fusobacteriaceae	1.744	2.414	0.24	5.62	6.15	0.39	6.14	0.30
f__Desulfovibrionaceae	1.466	2.029	5.64	0.11	1.12	0.65	1.66	5.80
f__Bacteroidaceae	1.407	1.948	4.88	0.15	0.00	0.00	0.28	5.49
f__Methylocystaceae	1.339	1.853	5.00	0.12	0.41	0.26	0.41	5.30
f__Streptococcaceae	1.317	1.823	0.13	2.31	0.23	6.70	0.39	0.16
f__Rhodocyclaceae	0.5333	0.7382	1.99	0.09	0.14	0.12	0.13	2.17
f__Acetobacteraceae	0.3631	0.5026	1.30	0.12	0.14	0.08	0.07	1.51
f__Conexibacteraceae	0.2751	0.3807	0.97	0.07	0.02	0.14	0.01	1.08

Note. Overall average dissimilarity = 46.08. DF1: Paddy field; DF2: Forest fire; DF3: Oil palm plantation; UF1: Sungai Karang Peat Swamp Forest site 1; UF2: Raja Musa Peat Swamp Forest; UF3: Sungai Karang Peat Swamp Forest site 2; Av. dissim: Average dissimilarity; Contrib.: Taxa contribution

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A Retrospective Study of Vertebral Fracture and Luxation in Dogs Presented to University Veterinary Hospital, Universiti Putra Malaysia in 2015 to 2017

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ABSTRACT

To date, there is data paucity on canine vertebral fracture and luxation (VFL) in the local setting. Therefore, it was hypothesized that the geographical location and stray dog population would influence VFL cases in the University Veterinary Hospital, Universiti Putra Malaysia (UVH, UPM). This study aimed to describe the incidence and characteristics of VFL in dogs presented to UVH, UPM. Medical records, including radiographic images of 74 dogs between 2015 to 2017, were reviewed. VFL dominated the spinal cord disease in dogs at 49% (n = 36/74), exceeding intervertebral disc disease (IVDD) and acute non-compressive nucleus pulposus extrusion (ANNPE). Half of VFL cases were contributed by intact male, small breed dogs aged more than one-year-old, with 52% (n = 11/19) of cases caused by vehicular accidents. Almost two-thirds (n = 21/36) of dogs with VFL were outdoor or stray dogs, and the Th3-L3 region was the most susceptible (52%, n = 19/36) for VFL. More than 70% (n = 25/36) of the patients had unstable fractures, highly associated with severity. In conclusion, the occurrence of VFL in UVH, UPM is three times higher than reported in western countries and most likely contributed by a large number of outdoor and stray dogs.

Keywords: Dogs, prognosis, thoracolumbar, vehicular accident, vertebral fracture and luxation

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INTRODUCTION

Vertebral fracture and luxation (VFL) is a common spinal cord disorder and constitutes about 7% of all spinal diseases in dogs (Bali et al., 2009). VFL may affect one or more vertebrae with common lesions reported at the dorsal spinous process, articular facets, and vertebral body (Denny & Butterworth, 2000; Dyce et al., 2009). Vehicular accidents and high-rise syndrome are major causes of VFL in companion animals represented at 40% to 60% (Jeffery, 2010). Fifty per cent (50%) of VFL cases in dogs occurred in the thoracolumbar area (Bali et al., 2009; Jeffery, 2010). It is due to the rigid attachment of the ribcage to the thoracic vertebrae through the costovertebral joint with a well-muscled but slightly mobile lumbar area, increasing the region's susceptibility towards VFL (Jeffery, 2010; Zotti et al., 2011). The clinical signs of VFL vary according to the site of the fracture and the degree of spinal cord damage (Hawthorne et al., 1999; Jeffery, 2010). The absence of deep pain perception always suggests a poor prognosis (Bali et al., 2009; Hettlich, 2017; Olby et al., 2003). Most patients are presented with acute and non-progressive signs. However, they may demonstrate progressive symptoms if the fracture is unstable or there is the presence of a haemorrhage (Jeffery, 2010).

The diagnosis of VFL can be achieved by radiograph and/or computed tomography (CT) scan. Plain radiography can detect VFL, but computed tomography provides superior three-dimensional images crucial in surgical planning (Hettlich et al., 2010; Lee & Thumbikat, 2015; Park et al., 2012). Magnetic resonance imaging (MRI) is preferred in assessing the intramedullary lesion and soft tissue structures in the adjacent area (da Costa & Samii, 2010; Gallastegui et al., 2019). Surgical repair is strongly recommended in patients with an unstable fracture with severe spinal pain and neurological dysfunction, although the absence of deep pain sensation tremendously affects the general prognosis (Gallastegui et al., 2019; Jeffery, 2010). Patients with 100% or more significant vertebral displacement and absence of deep pain have no hope for ambulation (Bagley, 2000; Olby, 2012; Platt, 2008).

The number of studies on VFL in companion animals is limited. The previous reports are centred in the United States, Canada, and European countries (Bali et al., 2009; Bruce et al., 2008; Hawthorne et al., 1999). However, no such report has been established in Asian countries. Therefore, this study aims to describe the incidence, distribution, and clinical characteristics of canine VFL in UVH, UPM.

MATERIALS AND METHODS

The medical records of dogs presented with spinal cord cases in UVH, UPM were reviewed between January 2015 and December 2017. Basic signalment data include age, breed, sex, body weight, and management. Age was recorded in years and categorised into young (< 1 year old), adult (1-5 years old), and senior (> 5 years old). While gender was classified into intact male, castrated male, intact female, and spayed female. The breeds were grouped

into small (1kg to 10kg), medium (10kg to 20kg), and large breed dogs (20kg to 50kg) (Shamir et al., 2002). The detail on the management, such as indoor, outdoor or stray, was also collected. Information on localisation and severity was documented based on neurological examination findings. The lesions were classified according to their location within the vertebral column (C1-C5, C6-Th2, Th3-L3, L4-L7, and S1-S3). Five-point grading system was utilised for each case: I = pain only; II = proprioceptive deficits and/or ambulatory para-/tetraparesis; III = non-ambulatory para-/tetraparesis; IV = para-/tetraplegia; V = para-/ tetraplegia with loss of deep pain perception (Sharp & Wheeler, 2005). All patients were offered routine diagnostics, including haematology and biochemistry analysis, survey radiography with and without CT or MRI.

A three-compartment model was used in this study to classify unstable fractures (Kinns et al., 2006; Lanz, 2003). The vertebra was divided into the dorsal, middle, and ventral compartments. The dorsal compartment comprises the articular processes, laminae, pedicles, spinous processes, and supporting muscles. The middle compartment includes the dorsal longitudinal ligaments, the dorsal aspect of the intervertebral discs' annulus fibrosus, and the vertebral body's dorsal part. The ventral compartment comprises the vertebral bodies, the lateral and ventral annulus fibrosus, the nucleus pulposus, and the ventral longitudinal ligaments. The fracture is considered unstable if the lesion involves more than one compartment of vertebrae. For VFL cases, cerebrospinal fluid analysis was not typically offered unless concurrent spinal cord conditions were suspected. All data recorded were obtained at the time when the initial diagnosis was made. The descriptive information was tabulated and analysed using GraphPad Prism 7.0 (GraphPad Software, United States of America).

RESULTS

A total of 122 spinal cord cases were presented to UVH, UPM from January 2015 to December 2017 and 74 cases fit the inclusion criteria. Among these cases, 49% (n = 36) was diagnosed with VFL through radiographs, CT or MRI, while other spinal cord disorders represented 51% of the cases; intervertebral disc disease (n = 32), acute non-compressive nucleus pulposus extrusion (ANNPE) (n = 2), one case each of fibrocartilaginous embolism, spinal cord neoplasia, discospondylitis, and subarachnoid diverticulum. Due to various reasons, including inadequate history and lack of diagnostic investigation, 48 cases were excluded.

The distributions of VFL cases according to the dog breed are presented in Table 1. Most patients were from local breeds (n = 14/36), followed by four cases from Shih Tzu and three cases from Poodle. Miniature Pinscher, Spitz, Siberian Husky, and Terrier dogs contributed eight cumulative VFL cases. The rest of the VFL patients were from various breeds, including Doberman, Chow Chow, French Bulldog, German Shepherd

Table 1

Breed distribution for vertebral fracture and luxation cases in UVH, UPM from January 2015 to December 2017

Breed	No. of cases	Percentage (%)
Local	14	38.9
Shih Tzu	4	11.1
Poodle	3	8.3
Miniature Pinscher	2	5.6
Spitz	2	5.6
Siberian Husky	2	5.6
Terrier	2	5.5
Doberman	1	2.8
Chow Chow	1	2.8
French Bulldog	1	2.8
German Shepherd Dog	1	2.8
Maltese	1	2.8
Pekingese	1	2.7
Pomeranian	1	2.7

Note. UVH = University Veterinary Hospital; UPM = Universiti Putra Malaysia

Dog, Maltese, Pekingese, and Pomeranian. The demographic characteristics of VFL are summarised in Table 2.

Most of the cases (n = 21) involved small dogs, whereas large dogs appeared to be the least affected group (n = 4). The majority of VFL cases were represented by senior (n = 17) and adult dogs (n = 15), and only four cases were young dogs aged less than one year old. Intact male dogs contributed to the highest number of VFL at 64% (n = 23), with two-thirds of the population coming from small breed dogs (n = 14). Both neutered female and male dogs were the least affected group, and they contributed to five cases in total. Furthermore, outdoor and stray dogs had a higher proportion of VFL at 58% compared to indoor dogs.

The number of VFL cases caused by vehicular accidents was 19, followed by 10 cases of high-rise syndrome, and five cases of dog bites (Figure 1). Stepping accident and pathological fracture due to underlying diseases contributed to two cases overall. More than 50% (n = 19) of the lesion occurred at the Th3-L3 vertebral region (Figure 2), with the most susceptible area found at the thoracolumbar junction (Th10-L2). In terms of susceptibility, L4-S1 (n = 8) and C1-C5 (n = 6) areas were recorded as second and third, respectively, after Th3-L3. A higher proportion (71%; n

= 17) of fracture cases affected more than one vertebral compartment with vertebral body lesion recorded as the highest (Table 3). VFL patients were commonly presented with grade 2 (n = 10, 28%) and grade 5 (n = 9, 25%) as shown in Table 4. All grade 5 patients had lesions at Th3-L3 with eight cases of unstable fracture. Among these cases, four patients had abnormalities in micturition.

Table 2

Demographic characteristic of vertebral fracture cases presented in UVH from January 2015 to December 2017

	Total	Small dogs (< 10 kg)		Medium dogs (10-20 kg)		Large dogs (> 21 kg)	
	n = 36	%	n = 21	%	n = 11	%	n = 4
Age (year/s old)							
Young (< 1)	4	14.3	3	9.1	1	0	
Adult (1-5)	15	38.1	8	54.5	6	25	1
Senior (> 5)	17	47.6	10	36.4	4	75	3
Sex							
Intact male	23	66.6	14	63.6	7	50	2
Intact female	8	19.1	4	36.4	4	0	
Neutered male	2	0		0		50	2
Neutered female	3	14.3	3	0		0	
Management							
Indoor	15	61.9	13	9.1	1	25	1
Outdoor and stray	21	38.1	8	90.9	10	75	3

Note. UVH = University Veterinary Hospital; n = Number of cases

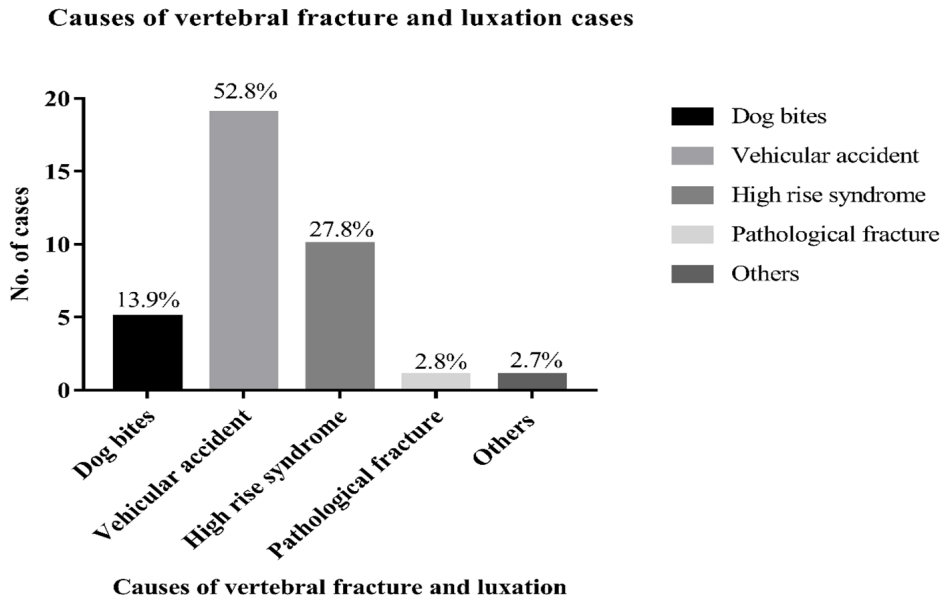

Figure 1. Causes of vertebral fracture and luxation cases recorded in UVH, UPM from 2015- 2017

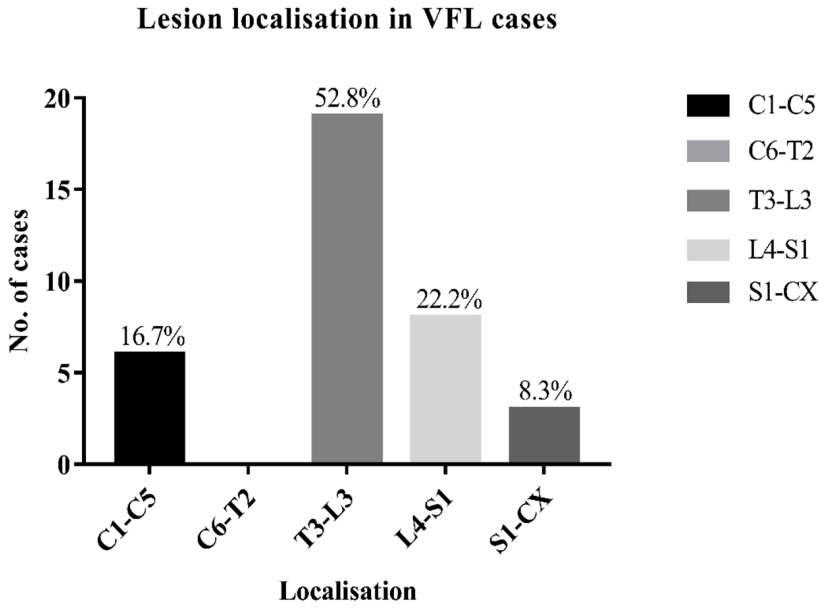

Figure 2. Localisation for vertebral fracture and luxation cases in UVH, UPM from 2015-2017

Note. VFL = Vertebral fracture and luxation; C = Cervical; Th = Thoracic; L = Lumbar; S = Sacrum; Cx = coccygeal

Table 3

Vertebral compartment involvement for fracture cases

Compartment affected (fracture cases only)		Percentage of cases (%)
Dorsal	Spinous process dorsal ligamentous structure, lamina, articular process, and pedicles	2.78%
Middle	Dorsal longitudinal ligament, dorsal aspect of vertebral body and annulus fibrosus and transverse processes	2.78%
Ventral	Ventral aspect of vertebral body and annulus fibrosus, nucleus pulposus, and longitudinal ligament	13.89%
More than one compartment		70.83%

Table 4

Neurological deficits grading

Grading		No. of cases	Percentage of cases (%)
1	Pain with no neurological dysfunction	6	16.67%
2	Proprioceptive deficit or ambulatory paresis or both	10	27.78%
3	Non-ambulatory paresis	8	22.22%
4	Plegia	3	8.3%
5	Plegia with loss of voluntary urinary function and loss of deep pain perception	9	25%

DISCUSSION

In this study, VFL was recorded as the most common spinal cord disease at 49%. This finding is inconsistent with previous reports where authors described IVDD as the most familiar spinal cord disease in dogs (da Costa & Samii, 2010; Denny & Butterworth, 2000). In two studies conducted in Canada and Switzerland, VFL was recorded between four to eight cases per year (Bali et al., 2009; Bruce et al., 2008) compared to 12 cases reported per year in UVH, UPM. The vast difference could be contributed by many outdoor and stray dogs in the present study area. The dogs admitted to UVH, UPM were mostly local breed and stray dogs; hence, it is expected to be overrepresented in this study. Despite various breeds in the study, the spinal anatomy between breed, size or age are comparable (Bodh et al., 2016;

Castañeda-Herrera et al., 2017); thus, it is less likely to affect the nature of the lesion. The extreme forces such as bending, torsion, and shear exerted to the vertebrae also play a significant role in VFL (Albernaz et al., 2016; Park et al., 2012).

Small breed dogs with VFL are present in a large proportion at 58%, and this finding may suggest that these dogs are susceptible to VFL. However, the data was mainly collected in a single institution surrounded by urban areas and could be overpopulated by small breed dogs. Likewise, dogs in adult and senior groups were prone to VFL in this study, consistent with a few studies (Bali et al., 2009; Bruce et al., 2008). Adult dogs are very energetic and still at the stage of exploring their surroundings (Hammerle et al., 2015). Their reactions towards stimuli are quicker than senior dogs. Therefore, it is not a surprise if they are susceptible to VFL. Senior dogs may have poor problem-solving. Their reaction time against stimuli may be slower than young dogs, making them vulnerable to accidents (Hammerle et al., 2015). In this study, puppies had the lowest incidence of VFL as most of them were confined in the house until they were trained.

The numbers of intact male dogs with VFL were higher compared to the neutered male and female dogs. Intact male dogs are aggressive, and their behaviours are usually driven by testosterone (McGreevy et al., 2018; Warnes, 2018). They are also fearless (Warnes, 2018), which may explain the high prevalence of VFL in this group. Furthermore, outdoor and stray dogs were prone to suffer from VFL as these dogs spent most of their time outside without supervision. Stray dogs contributed nine cases, and they are always presented with multiple injuries due to accidents, which increased the incidence of VFL in the outdoor group. More than 50% of VFL cases were caused by vehicular accidents, as previously reported in other studies (Bali et al., 2009; Di Dona et al., 2016; Olby et al., 2003). High-rise syndrome came second after vehicular accidents, which was most likely contributed by dogs living in condominiums and apartments. Five out of 36 VFL cases were caused by dog bites, which is in line with previous studies where dog bites were mentioned as one of the causes of VFL, but no prevalence was provided (Bali et al., 2009; McKee, 1990).

In this study, most VFL cases concentrated at the Th3 to L3 region, specifically at the thoracolumbar region (Th10-L2). The thoracolumbar region is recognised as the most mobile junction between the constrictive thoracic part and rigid lumbar part in any dog, making the region more susceptible to VFL (Bali et al., 2009; Jeffery, 2010). The cranial thoracic is a very rigid part due to support from the costochondral junction and ribcage (Jeffery, 2010), which might explain the lack of cases affecting this area. The least affected site was the sacrococcygeal region with three cases only. The area is more associated with cat VFL cases than dogs (Davies & Walmsley, 2012; Jeffery, 2010). The cats' small size causes them to be entrapped under vehicles during an accident, thus increasing the risk for tail traction instead of dogs.

The results revealed that almost three-quarters of fracture cases were classified as unstable via radiographs. Among these numbers, most of the cases were caused by vehicular accidents, suggesting a high association between vehicular accidents and instability. In addition, classification via radiographs may not be accurate as CT diagnosis is known to be more sensitive in determining the compartment involved, fracture fragments within the vertebral canal, subluxation, and vertebral canal narrowing (Kinns et al., 2006).

Standard neurological grading for thoracolumbar was used to classify the severity in this study. Most VFL patients were presented with grade 2 and grade 5 at 27% and 25%, respectively. Almost 90% of grade 5 patients had fractures in more than one compartment, suggesting that unstable fracture is likely associated with lesion severity. Furthermore, micturition problems are commonly seen in patients with severe grades (Bagley, 2000; Jeffery, 2010; Olby et al., 2003), supported in this study. Patients with urinary problems tend to have a poorer prognosis. However, unfortunately, this study lacks information to corroborate the previous findings (Bagley, 2000; Jeffery, 2010; Olby et al., 2003). Most grade 5 patients were euthanised 24 to 48 hours after losing deep pain perception (Bali et al., 2009; Bruce et al., 2008; Duval et al., 1996).

CONCLUSION

In conclusion, VFL is the most common cause of spinal cord disease in dogs presented to UVH, UPM. A large population of local and stray dogs contribute to VFL, with 50% of lesions occurring at Th3-L3. Most of the patients with unstable fractures suffer from severe lesions that carried a poor prognosis. The characterisation of VFL cases in this study can assist in refining the medical and surgical treatment to improve the clinical outcome of VFL cases. However, this data is still limited to a single institution, and the present results should be applied cautiously to other settings.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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Review article

The Potential of Silicon in Improving Rice Yield, Grain Quality, and Minimising Chalkiness: A Review

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ABSTRACT

Silicon (Si) is a micronutrient that can increase the resistance of certain plants against multiple biotic or abiotic stresses. It is known that Si has a beneficial effect on plant growth, beginning in the soil, which could lead to a good crop yield. Despite its benefits, Si is not listed among the generally essential elements or nutrients for rice production in many countries such as Malaysia. This review discusses the ability to uptake Si and its benefits on rice. Environmental factors affect rice production, and among the factors, high temperature has been shown to disrupt the physiological development of rice grain, which contributes to chalkiness. Chalkiness is an undesirable trait that decreases grain's value, milling, cooking, and eating quality. The application of Si could ameliorate rice grain quality, thus providing a valuable reference for Si fertiliser use in high-quality rice production. This review also presents an update on the potentials of Si in improving the rice yield and grain quality, including Si's ability to minimise grain chalkiness. Therefore, it is anticipated that Si applications will increase rice yield and grain quality and help to reduce chalkiness.

Keywords: Chalkiness, high temperature, rice quality, rice yield, silicon fertilisation

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INTRODUCTION

Rice (*Oryza sativa* L.) has fed more people in history than has any other crops. It serves as the primary source of dietary energy. It has a good proportion of proteins, vitamins, and minerals, thus providing the calorie requirements of the global population. Its indubitable importance has led to its extensive cultivation globally. In fact, it has become the second-highest cultivated cereal after wheat (Rajamoorthy et al., 2015). As a result, rice is a real food for over half of the world's population. The annual world rice production (based on milled rice) has increased from approximately 448 million metric tonnes in 2008/2009 to more than 496 million metric tonnes in 2019/2020, as reported by the US Department of Agriculture (2021). By 2025, approximately 800 million metric tonnes of rice, a 25% increase, would be needed to meet the requirements of the human population.

In Malaysia, rice is an important crop. It is recognised as the third most widely planted after oil palm and rubber. Rice cultivation has always received particular attention in the effort to ensure the nation's food security. Rice demand is expected to continue with an increasing trend over the coming years due to the continuing urbanisation and increment of the global population. The Malaysian government has implemented various protection policies to ensure rice sustainability while attaining optimum self-sufficiency levels (SSL). The present rice SSL in Malaysia is around 72%. Peninsular Malaysia has eight regions of granaries dedicated to ensuring rice SSL for the nation. Among these regions, northwest of Selangor is known as the most productive rice-growing area nationwide, hence being nicknamed 'The Rice Bowl of Selangor'. The area covers 36,602 ha with an average paddy yield of 4.8 t/ha and 174,088 tonnes of paddy production in 2019 (Ministry of Agriculture and Food Industries Malaysia [MAFI], 2019). Located in the west coast zone, northwest of Selangor experiences dry periods in February, June, and July.

Over 90% of rice is grown and consumed in Asia, which has the world's largest population. Rice cultivation mostly takes place in areas where the temperatures are already near-optimal for rice production. However, rice production is exceptionally vulnerable to environmental changes and severe conditions such as drought and flooding, which are issues in Malaysia. Consequently, any further increases in average temperatures or high temperatures above the optimal, particularly during reproductive and grain-filling periods, may reduce grain yields.

After milling, the physical appearance of grain is a significant determinant of consumer preference as rice is sold in milled form. These qualitative properties are readily attainable in many areas of the world. Although quality characteristics can be subjective and conflicting, the general population prefers rice with a uniform shape and translucent endosperm. Rice grain is thus termed chalky when it is not perfectly translucent in appearance. This trait, together with head yield, is the key characteristic used in determining the rice

quality and its price. Chalkiness occurs mainly at the rice grain's central area, where at least half of the grain area is opaque white (Lisle et al., 2000). When this occurs, breakage during milling will ensue. As the proportion of chalky grains reaches 15%, the quality of rice also decreases (Kim et al., 2000). Additionally, milled rice containing over 2% chalky grains is not accepted in certain regions (Lisle et al., 2000). Therefore, minimising chalkiness is a viable strategy for producing more head rice. Every 1% decrease in chalkiness is accompanied by a 1% increase in head rice yield (Zhao & Fitzgerald, 2013).

Silicon (Si) is generally the most abundant element in the earth's crust. It can be absorbed by plant roots in large amounts, thus resulting in positive effects if applied to the soil where rice and many other crops are grown. Furthermore, Si is an element that improves resistance to multiple stresses, including biotic and abiotic stresses. Thus, it can stimulate plant growth while not causing harm, corrosion, and pollution, even in excess application. Several researchers had studied and demonstrated the improvement in physiological characteristics when Si was applied during planting (Liang et al., 2007). Although deemed a non-essential element for the growth of higher plants, Si seems to benefit certain plants, particularly in plants under stress conditions. Furthermore, many soils, including subtropical and tropical soils, are typically low in Si availability, so that supplemental Si will benefit both soil and cultivated crop. In the last 20 years, significant findings on the advantages of Si in several countries have helped develop Si fertilisation as an agronomic practice for several crops worldwide. Nonetheless, very little information on the application of Si in Malaysian agriculture is available. Therefore, this review aims to present an update on the potentials of Si on improving the rice yield and grain quality, including Si's ability to minimise grain chalkiness.

Si Uptake and Deposition in Rice Plant

After oxygen, Si is the second most abundant element, comprising approximately 29% of the solid earth's crust (Haynes, 2014). Si occurs not as a free element but bonded with other elements to form chemical compounds, such as silicon dioxide (SiO_2) (Heckman, 2012). Generally, Si is abundant in the soil as mineral quartz and clay, but its abundance in a soluble form (silicic acid; H_4O_4Si) is highly variable. Silicic acid is absorbed by plants to be continuously transformed into insoluble polymers (Epstein, 1999; Ma & Takahashi, 2002). Si can be added through irrigation water and fertilisation to improve the soil's physical, chemical, and biological properties. However, continuous cropping of land, natural weathering, or inherently deficient soils can be causes of Si deficiency. Si, as a fertiliser, plays various essential roles in plants' mineral nutrition. It provides a nutrient beneficial for the robust and competitive growth of many crops, including rice (Ma & Yamaji, 2006).

Si in the soil can be absorbed by plants largely by roots and directly affect plant development. However, the total elemental percentage of Si in the soil is not what

the plant can utilise. Plant roots take Si as silicic acid (soluble molecule) mediated by transporters and translocated to leaves and shoots in the same form. The uptake of Si by the roots is facilitated by a type of transporter, namely Low silicon rice (Lsi). *Lsi* gene is responsible for controlling Si accumulation in rice. The absorption of Si in rice is mediated by transporters, Lsi1 and Lsi2 in roots and Lsi6 in shoots (Ma & Yamaji, 2008). The uptake and transport of Si are illustrated in Figure 1.

Rice is a Si accumulator and requires a high amount of Si for stable, vigorous growth, and increased production (Ma & Takahashi, 2002; Meena et al., 2014). Rice plants can take up Si in the range of 230 to 470 kg/ha (Ma & Yamaji, 2006). Over 90% of Si in the soil is taken up by roots and translocated to aerial plant parts (Ma & Takahashi, 2002). Plants vary significantly in Si accumulation because of different abilities between different species to take

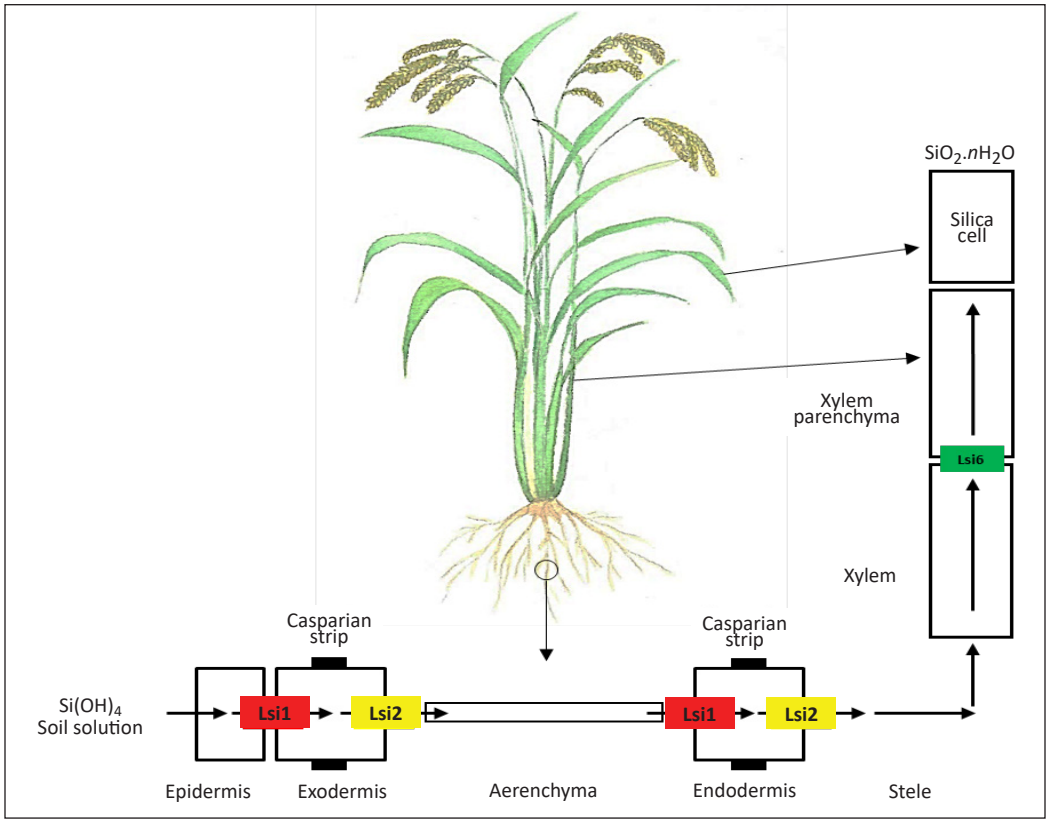

Figure 1. Si uptake system and transportation across the rice plant, mediated by Si transporter. Plant root takes up Si (as silicic acid) from soil solution through the root epidermis cell and transports it to exodermis cells by the passive Lsi1 and the active Lsi2. In the aerenchyma, Si moves apoplastically until it reaches the endodermis, where the Lsi1 and Lsi2 load Si into the stele. Si enters the xylem, mediated by an unknown transporter, travels further up into the leaves and shoots. Lsi6 is responsible for unloading the Si into the xylem parenchyma. Si is formed as amorphous silica ($SiO_2.nH_2O$) in the cell walls of shoots and leaves, called silica bodies or silica cells. Modified with reference (Ma et al., 2011; Ma & Yamaji, 2015)

up Si by the roots. Plant species with a Si content of more than 1.0% of dry weight are considered Si accumulators. In comparison, those with a range between 0.5 % and 1.0% are considered intermediate species, and those with less than 0.5% are considered Si excluders. In higher plants, the highest Si accumulation in Gramineae is found in rice (Hodson et al., 2005) as Si is accumulated at up to 10% of shoot dry weight. This amount is higher than that of essential nitrogen (N), phosphorus (P), and potassium (K) macronutrients (Ma & Yamaji, 2008). Therefore, Si has been used in increasing nutrient availability such as N, P, K, calcium (Ca), magnesium (Mg), sulphur (S), zinc (Zn), and decreasing mineral stress, such as nutrient toxicity of iron (Fe), aluminium (Al), manganese (Mn), cadmium (Cd), and arsenic (As) (Ma & Yamaji, 2006).

A high level of Si uptake in rice is related to the superior ability of roots to absorb Si, much faster than that of water, and readily understandable by transpiration. Rice exhibited much higher Si uptake than other gramineous species like maize, sorghum, wheat, and barley (Ma & Yamaji, 2006). More than 90% of total Si in the shoots exist as silica gel in the epidermal cell wall of leaves, stems, and hulls, forming a Si-cuticle double layer and a Si-cellulose double layer (Ma & Takahashi, 2002). Si accumulation in cell walls improves their strength and rigidity, thus consequently increasing rice resistance to multiple threats, such as diseases, pests, and lodging (Ma, 2004; Ma & Takahashi, 2002).

Chalkiness Reduces Rice Quality and Acceptance

Physically, chalky rice is more susceptible to breakage and reduces head rice recovery, consequently reducing millers' market value and economic returns. Along with head rice, chalkiness is used to determine the quality and price of rice in the markets. Chalky kernels have lesser starch granules density than translucent (unchalky) kernels, rendering them prone to breakage during milling (Ebron, 2013). In addition, chalky endosperm has a different cellular morphology in which starch granules do not develop adequately. The remaining air spaces between the granules reflect light, rendering the grains less translucent or chalky (Kim et al., 2000; Lisle et al., 2000; Zhaomiao et al., 2015).

Rice chalkiness formation is a complex physiological process closely associated with the biosynthesis and accumulation of starch in the endosperm. The physiological causes of chalkiness could be insufficient nutrient supply to develop endosperm, reduction of starch synthesis capabilities in the endosperm, and degradation of starch during ripening (Yamakawa et al., 2007). Great attention has been focused on the study of starch and amylose. Amylose content is the foremost criterion of grain quality used to indicate rice starch quality. Chalky rice tends to be low in amylose but high in amylopectin compared to translucent rice (Lisle et al., 2000; Patindol & Wang, 2003). The quality differences between chalky and translucent rice in texture as well as cooking and eating quality of cooked rice have been

studied (Chun et al., 2009). Chalky rice is softer than translucent rice and can easily absorb water during cooking. Due to the loosely packed starch granules, its volume expansion ratio is slightly higher than that of translucent rice. The low amylose content of chalky rice results in a more soluble solid, thus leading to its lower eating quality. The lower gelatinisation indicates a more short-branched amylopectin structure in chalky grain. The sensory analyses on cooked chalky rice revealed lower quality and acceptance (Chun et al., 2009).

All the physical appearance, milling properties, nutritional value, and cooking quality are related to the content and composition of starch and protein in the endosperm. Like starch, protein is another main component of rice endosperm. Therefore, it is also the key to determining the rice's nutritional value. Furthermore, the protein content can affect the cooking quality as protein-starch interaction can impede the starch gelatinisation and disruption of the structure of the protein during cooking increases the viscosity of cooked rice (Yu et al., 2008).

Temperature Affect the Rice Yield and Quality

Rice is a tropical cereal, but it is also grown in some temperate zone where the average annual temperature is not extreme. The quality of rice grains during the growing season is affected by genetic and environmental conditions. Global warming and climate change threaten not only the rice yield but also its quality. The frequency and intensity of drought, together with rising temperatures, pose a severe challenge to rice production. High temperatures, especially during grain filling, can cause significant damage to rice grain quality. Continuous cloudy weather or rainfall, particularly during grain-filling, often results in significant yield losses and poor grain quality. The grain-filling stage is considered vulnerable when exposed to extreme conditions, especially high and low temperatures. The other environmental elements include solar radiation, atmospheric carbon dioxide, light, water, and soil nutrient (Patindol et al., 2015).

Due to global warming, rice production and grain quality characteristics have been influenced, for example, when temperature increases over a certain threshold level for a particular duration which is sufficient to cause unalterable damage to plant growth. For each degree Celsius increase in global mean temperature, there is a decrease in rice yield. Research by Gumel et al. (2017) on the impact of climate change in Malaysia, particularly in granary areas of Muda Agriculture Development Authority (MADA), Kemubu Agriculture Development Authority (KADA), and Integrated Agricultural Development Area (IADA) showed that an increase of temperature above the optimal level for rice production has contributed to yield reduction. They revealed that an increase of 1°C in the maximum temperature caused a decrease in yield from 0.2% to 4.5% for MADA and KADA during the mian season

and 0.5% to 2.3% for MADA and IADA during the off-season.

Al-Amin et al. (2010), in their study on climate scenario in Malaysia, predicted that yields of rice would fall between 4.6% and 6.1% due to a rise of 1°C in temperature at the current level of CO_2 concentration, with the probability of a further decrease in yield with increasing temperature. With this scenario, they projected that by 2060, rice productivity would be reduced by 34.8% per hectare. This prediction was supported by Vaghefi et al. (2013), who studied the effects of climate changes on rice yield for both main and off-seasons during 2013 until 2030 in eight main granaries in Peninsular Malaysia. Based on predicted weather and crop management practices, an expected increase of temperature and rainfall throughout the growing period would reduce the rice yield by 18.6% for the main season and up to 45.5% for the off-season. These findings showed that rice yield would be more negatively affected by climate change during the off-season than the main season, which could be explained by differences in minimum and maximum temperatures and rainfall between the main and off-season. The maximum and minimum temperature and rainfall were projected to increase about 0.05°C and 0.12 mm per year, respectively, during the main season. However, during the off-season, the minimum and maximum temperatures were projected to increase about 0.19°C and 0.08°C per year, respectively. At the same time, rainfall would decrease by about 0.18 mm per year.

Global warming impact yield and facilitate the chalky formation and affects the milling quality of rice (Ishimaru et al., 2009; Radziah et al., 2010). High-temperature stress during grain filling boosts the formation of chalky grains, resulting in irregular-shaped amyloplasts, which consequently lead to breakage during milling (Wei et al., 2010; Yamakawa et al., 2007). According to Cooper et al. (2008), rice grain quality is significantly reduced by the high temperature of 35°C if exposed longer than five days during grain filling. They observed that rice grain quality is good at the optimum temperature of 22°C to 25°C during grain filling. With a rise in nighttime temperatures, the chalky kernels increased. However, in field conditions of four dry and wet seasons, Zhao and Fitzgerald (2013) reported that high temperature as a single parameter does not lead to chalkiness. They suggested that other than high temperature, climatic parameters like relative humidity (RH) and vapour pressure deficit (VPD) in combination might also be contributing. Low RH and high VPD in the dry season resulted in low chalky rice and high head rice even with the maximum temperature. However, in the wet season with the opposite condition, the chalky grain was high with a lower head rice yield.

The formation of kernel chalkiness is associated with the kernel development within the endosperm, which includes the synthesis of starch and the structure and arrangement of starch granules (Yamakawa et al., 2007). According to Kaneko et al. (2016), high temperature has

increased soluble starch content in chalky grains. However, those in translucent grains were not changed, regardless of environmental stress. These indicate that starch degradation, instead of starch synthesis, is involved in chalky grain formation at the grain-filling stage and is influenced by the high-temperature stress. High temperature can also decrease the level of metabolic compounds associated with starch biosynthesis; hence starch production in the endosperm declines (Cao et al., 2009). Additionally, the amylose content tends to decrease, increase, or remain depending on different varieties. Grains at high temperatures have lower compactness of amylose (starch granules) (Cheng et al., 2005) as compared to low temperatures (Cooper et al., 2008).

Under optimum conditions, the edible part of rice grains, the endosperm, comprises starch granules yielding hard vitreous translucent grains. However, starch synthesis is weakened in ascertain conditions and results in less starch per endosperm or smaller starch granules. Some enzymes involved in starch biosynthesis are susceptible to a temperature above optimal conditions, affecting starch deposition (Fitzgerald & Resurreccion, 2009). For example, heat stress during grain filling can increase chalkiness due to increased activity of α-amylase, the hydrolysing starch enzyme. The enzymatic activity and gene expression of α-amylase increased more than twofold in response to increased temperature during the ripening period. In contrast, the expression of many genes involved in starch biosynthesis was regulated, preventing starch accumulation (Yamakawa & Hakata, 2010).

The Potential of Si in Improving Rice Yield and Chalkiness

Research on Si started in Japan at the beginning of the twentieth century when Si content was lower in rice leaves infected with blast disease. The relationship between Si and rice blast disease was studied extensively several years later until they realised that silicate application could increase the rice resistance to blast disease. Numerous studies were conducted to discover the physiological role of Si. In the 1930s, Si became significantly crucial for the growth and stability of rice production (Ma & Takahashi, 2002). However, Si was not applied in the rice field since Si was naturally abundant in the soil. Therefore, they assumed that the application of Si to the soil was unnecessary. Moreover, at that time, specific Si fertilisers were also not available. The application of Si on rice fields started after the Second World War when a special project was implemented to improve degraded paddy soils due to a food shortage (Ma & Takahashi, 2002). Si source, a by-product from the iron industry (calcium silicate slag), was used. Results showed that Si application helped to enhance productivity in declined paddy soils.

Many field trials have shown that Si as silicate fertiliser, either as organic or inorganic silicate, could significantly increase rice yield. Therefore, organic silicate materials such as rice straw and

silicate slag are widely used in many countries as alternative materials for inorganic silicate fertilisers (Ning et al., 2016). In addition, rice husks obtained from the rice-milling industry have been used as a source of Si in some countries like Japan. Nevertheless, this is not a standard practice due to the complex decomposition in the soil.

Si fertiliser has been reported to have a beneficial role in enhancing rice yield (Agostinho et al., 2017; Cuong et al., 2017; de Oliveira et al., 2019; Emam et al., 2014; Guntzer et al., 2012; Li et al., 2020; Liang et al., 2015; Siregar et al., 2021). In terms of yield components, Si improved the number of spikelets per panicle, spikelet fertility, and grain weight of rice (Ma et al., 1989; Takahashi, 1995). Furthermore, in the early 1960s, Okuda and Takahashi (1961) revealed that Si application had improved the plant height, grain weight, and Si uptake when Si was applied at later growth stages (after panicle initiation stage) compared to earlier growth stages. These indicated that Si application is more crucial at later growth stages rather than earlier stages. Furthermore, the addition of Si at the reproductive growth stage increased the number of spikelets, the percentage of ripening, and the grain weight (Nhan et al., 2012).

It is estimated that a rice crop that produces a total grain yield of 5 tan/ha can remove Si from the soil at a rate of 230 kg/ha to 470 kg/ha (Savant et al., 1997). Thus, Si may become a yield-limiting factor for rice production, necessitating exogenous Si fertiliser for an economical and sustainable rice production system. Calcium silicate slag is commonly applied by growers at 900 kg/ha of Si up to 1500 kg/ha has been shown to increase rice grain yields (Snyder et al., 1986). Si incorporated before seeding at 1000 kg/ha showed that Si alone and with fungicide increased the yields by 28% to 51% over the control (Seebold, 1998). However, recently Si fertilisation using chemical silicate at a rate of 100 kg/ha to 250 kg/ha offers promising results to improve rice yield (Babu Rao & Sushmitha, 2017). Therefore, the Si recommendation for rice varies between this range or higher, up to 500 kg/ha depending on its chemical and physical nature and soil factors (Cuong et al., 2017; Han et al., 2018; Jafari et al., 2013; Patil et al., 2017). Calcium silicate is one of the most regularly used Si fertiliser incorporated into the soil before planting.

The role of Si in improving resistance against various stresses has been discussed thoroughly (Ma, 2004). In the meantime, other researchers (Adrees et al., 2015; Sahebi et al., 2015) have illustrated several factors affecting Si uptake and deposition in plants. Mechanisms of Si on plant improvement are associated with the plant stress responses against various environmental conditions (Figure 2). Thus, when a plant detects some form of stress, Si can effectively activate natural defence mechanisms by triggering a wide range of plant responses involving molecular and cellular processes.

Si plays a role in controlling the nutrition of rice minerals that are essential for rice growth and development. Its benefits include

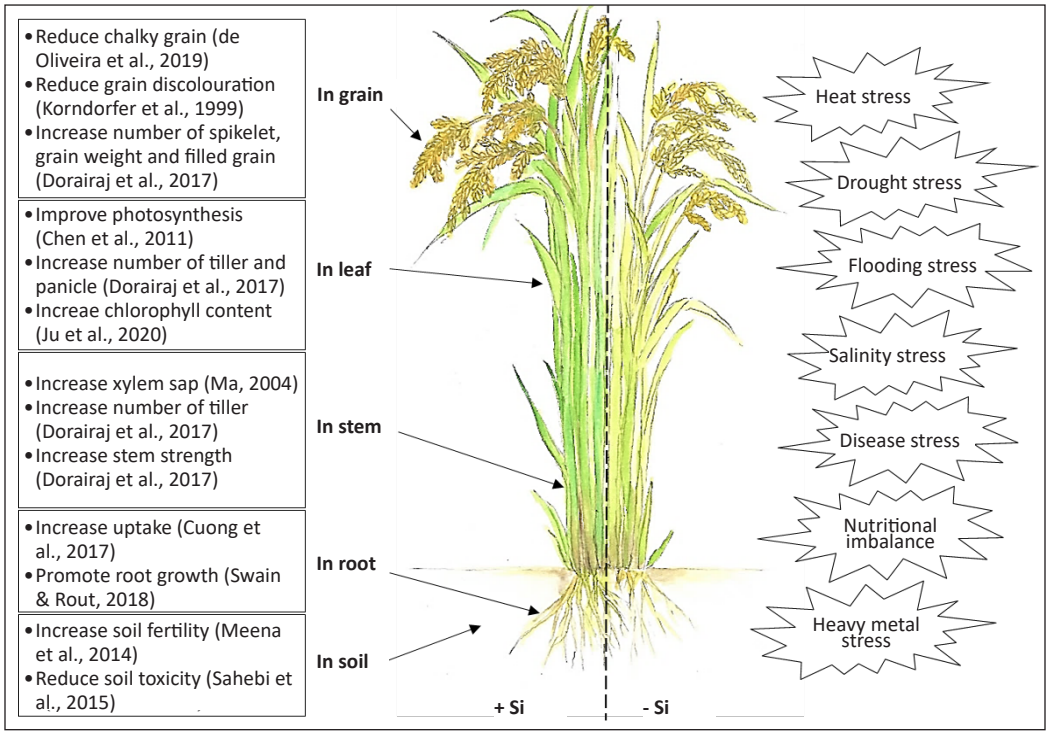

Figure 2. The hypothetical mechanism for Si-sufficiency and Si-deficiency in rice plant

improving fertiliser uptake efficiency, alleviating mineral toxicity (Sahebi et al., 2015), facilitating photosynthesis (Lavinsky et al., 2016), preventing lodging (Epstein, 1999), alleviating water (Agarie, 1998), enhancing the rice to be more resistant against diseases, and protecting rice against insect pests (Cooke & Leishman, 2011; Liang et al., 2007). Si deficiency might expose the rice plants to all these issues, thus limiting the rice yield to increase further. Datnoff et al. (2001) reported that low Si in soils tends to lower other essential nutrients. This positive effect was attributed to the corrective action of Si amendments and the increased availability of Si, P, Ca, and Mg to plants. In addition, Si can promote the oxidation power of root, and improve the soil condition, thus minimising the toxicity of heavy metals like Fe, Al, and Mn.

Additionally, Si can alleviate the imbalance in soil nutrients by increasing the pH and solubility of soil and increase the availability of phosphorus (P) and nitrogen (N) through the formation of silicified tissue in plants (Sahebi et al., 2015). The damaging effects of excess soil nutrients can also be alleviated by decreasing its extreme absorption. Si absorption in endodermal root cells can serve as an apoplastic barrier to reduce the uptake of soil nutrients by roots.

Si deposited in the plant cells forms a thick silicated layer on the leaf surface, thus alleviating water stress and reducing the loss of water transpiration. It also dramatically improves the light distribution within

the canopy by keeping the leaves, stems, and culms of plants more erect (Epstein, 1999). Ma (2004) reported a decrease in self-shading and photosynthesis rate when leaves are more erect, particularly during grain filling related to starch accumulation in grain. Accumulation of Si increases the stem strength by increasing the thickness of the culm wall and the number of vascular bundles, thus resulting in a decrease in lodging. The deposition of Si in the leaf epidermal cells and cell walls of hulls creates a double layer of silica cuticle and silica-cellulose on the surface of leaves, stems, and hulls. The impregnation of the cell wall with the Si particle improves the mechanical properties of plant tissues. It helps plants to bear various stress conditions. It decreases transpiration from panicle, thus reducing water loss so that the plant can withstand water deficiency and strong wind (Ma et al., 2001, 2002). Si is efficient in reducing the rate of transpiration from the hull by maintaining a high moisture condition within the hull.

In response to insect pests and pathogen attacks on the plants, various defence mechanisms activated or induced by Si application. Si has been shown to increase the resistance against diseases and pests by forming physical barriers on the tissue surface. The physical mechanism embeds Si deposition beneath the cuticle impeding pathogens entry and feeding activity of the insect pests. The biochemical defence may involve stimulation of defence-related enzymes, which results in increased expression of defence genes, resulting in decreased tissue damage by herbivory and pathogen movement within plant tissues. Molecular defence modulates transcriptomic and proteomic regulations that improve plant resistance against insect pests and diseases (Islam et al., 2020). Si deposition enhances plant resistance against infection when Si is absorbed by the roots (Datnoff et al., 2007). Farnaz et al. (2012) have evaluated the effectiveness of Si between granular silica gel, which was applied directly to soil compared to liquid sodium silicate, which was applied as a foliar spray and tested on rice variety MR 219. They reported that supplying Si through the roots yielded higher Si content in the rice leaves than foliar application, consequently contributing to better protection against blast infection. NurulNahar et al. (2020) have reviewed options for controlling rice blast diseases. Instead of using a chemical fungicide as the last option, they suggested Si as one of the practical and sustainable methods. However, these approaches are still not commonly used in local rice cultivation.

Research has shown that Si also increases rice grain quality, resulting in higher grain-milling quality and greater whiteness per grain (Prabhu et al., 2012). It is known that milling quality can be affected when grains are chalky and immature since they are easy to crack during polishing resulting in decreased rice-milling recovery. According to Alvarez et al. (2004), Si application also offers a better grain appearance besides producing a healthier plant. Reasonable rates of Si can decrease the chalky grain and increase protein content

and gel consistency (Zhang et al., 2007). In addition, Si is responsible for the formation of hulls, which can influence grain quality (Ansari et al., 2016). After leaves, large amounts of Si are deposited in the hulls as amorphous SiO_2. Most of the beneficial effects on grain quality are attributed to Si deposition in the cell walls of hulls. According to Ma and Yamaji (2006), the content of Si rice was 8.05% in hulls, and 4.21% in leaves, while rice with low levels of Si only had 1.44% and 0.48% in the hull and leaves, respectively. Plants that can absorb and accumulate a large amount of Si are beneficial because this element can increase stress-resistant, particularly for Si-accumulator plants, thus enhancing grain quality. According to Mizuno (1987), the poor quality milky-white grains exhibited low Si content in the hulls, parallel with the Si content in the straw. The involvement of Si affected the cell wall formation in the spikelet before heading (Inanaga et al., 2002). Research by de Oliveira et al. (2019) has revealed that Si fertilisation, combined with fungicide application, can reduce the percentage of chalky kernels and vitreous grains of rice cultivars sensitive to rice blast.

In terms of nutritional quality, adding Si as fertiliser could increase the protein concentration and mineral elements (Zn, Ca, Mg). Application of Si at rice booting stage could increase the concentration of most of the amino acids in brown and milled rice of Korean japonica rice (Q. Liu et al., 2017). X. Liu et al. (2020) also proved that Si, together with selenium (Se), could increase the protein content of hybrid rice in China, besides increasing the rice yield by 17.15% to 25.45%. Research by Mo et al. (2017) showed that Si fertiliser had improved the yield and aromatic characteristics in high-quality aromatic rice in Hunan, China. The content of 2-acetyl-1-pyrroline (2-AP), the principal aromatic compound that contributes to the aroma character in fragrant rice, has also increased. These valuable discoveries serve as a guideline for applying Si fertiliser to produce high-quality rice.

CONCLUSION

Silicon (Si) has been shown to significantly improve the soil structure, increase the efficiency of NPK fertilisers, alleviate the toxicity of metals, enhance the plant's resistance against diseases or pests and other environmental stresses. All of these factors can contribute to an increase in yield. Si has also been proven to improve the milling quality, grain quality both physically and chemically, and minimise chalkiness. Other than the strategy of developing new varieties with a reduced chalky grain percentage, the chalkiness problem can also be eliminated by adding Si as a nutrient to the plant. Therefore, adding Si as fertiliser during planting would be an effective way to improve the rice yield and grain quality, including minimising grain chalkiness. The reduction of chalky grains can also ensure the production of good quality rice grains. The proper of Si rate, time and application method should be studied and implemented for Malaysian rice varieties and then integrated into the rice cultivation systems.

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An Optimised TRIzol-based Protocol for the Improvement of RNA Extraction Yield of Tomato Stem

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ABSTRACT

One of the most common methods for purifying RNA is using TRIzol reagent because of its simplicity and economic feasibility. However, the drawback of this method is frequently the low quality of extracted RNA due to contaminants from the residue of phenol and guanidinium thiocyanate from the reagents. This study aimed to evaluate the improvement in the quality and concentration of RNA after the optimisation treatment. One-month-old tomato (*Solanum lycopersicum*) stem was used in this research. TRIzol or acid guanidinium thiocyanate-phenol-chloroform-based method was given optimisation treatments of the initial sample amount, twice chloroform extraction, overnight precipitation at low temperature, and three times final washing with ethanol. The results showed no significant improvement ($p > 0.05$) in the purity ratio A260/A280. At the same time, there was a significant improvement ($p < 0.05$) in RNA yield and purity ratio A260/A230. The quality of RNA was verified using agarose-formaldehyde electrophoresis gel. Eight of nine samples (89%) from the optimised group had better RNA integrity characterised by sharp bands for 28S and 18S

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rRNA. Furthermore, a representative sample from the optimised group was successfully synthesised into complementary DNA by reverse transcriptase-polymerase chain reaction (RT-PCR) with primers of the ubiquitin (UBI3) gene. To sum up, optimised TRIzol-based protocol provides meaningful insight to produce RNA with better quality and suitability for downstream applications.

Keywords: RNA extraction, RT-PCR, *Solanum lycopersicum*, TRIzol

INTRODUCTION

Tomato (*Solanum lycopersicum*) is an important food crop that provides nearly 11% of daily vegetable calories consumed per capita in 2003 (Liedl et al., 2013) and has consistently increased in the last decades. United States Department of Agriculture reported tomato per capita availability reached an average of 20.7 pounds by 2010-2017 (Baskins et al., 2019). However, not a part of the primary source of calories, scientific interest in tomatoes peaked after its development as a genome model of the Solanaceae family, containing many other important plant species such as potatoes and chillies. Various molecular investigations such as quantitative trait mapping, mutant screening, and gene expression analysis in tomatoes began to be carried out in the early 20th century (Giovannoni, 2004). One of the essential investigations is the molecular analysis of plant response to the environment, such as drought stress (Zhou et al., 2019), pathogen attack, bacterial wilt (Hyakumachi et al., 2012), and Fusarium wilt (Ferniah et al., 2015; Jaiswal et al., 2020). Analysis of plant responses in various stress conditions is studied through gene expression in plant organs, including stem organs.

The difficulties of research in gene expression are due to the low quality and quantity of RNA products from stem organs. On the other hand, plant stem is a resource of molecular analysis underlying plant defence as some of the most destructive plant diseases target the vascular system, specifically the xylem (Yadeta & Thomma, 2013). Purification of RNA from stem organs has its challenges because of the fibrous nature of the tissue that also contains many secondary metabolites such as polyphenols, polysaccharides, and various other compounds that can affect the final quality of RNA (Farrell Jr., 2017; Rajakani et al., 2013). For example, Wang et al. (2009) stated that RNA extraction of the tomato plant from various organs using a guanidinium thiocyanate-phenol-chloroform-based method showed lower RNA yield from stem than other organs such as leaves and roots. The low yield of RNA was presumably due to stems of tomatoes containing high content of polysaccharides and lignin. In addition, the stem of the tomato plant has an average fibre length of 980 µm, which is equivalent to other hardwood plants that range between 500 and 3000 µm (Fengel & Wegener, 1984; Uner et al., 2016).

Several studies using RNA of tomato stem that has been successfully carried out

for molecular analysis applications require spin column-based extraction kits, which are relatively expensive (Ishihara et al., 2012; Milling et al., 2011). However, the quality of the RNA itself is not reported. Another standard method is using monophasic reagent based on guanidinium thiocyanate-phenol-chloroform, which has now been packaged in commercial forms such as TRIzol (Invitrogen, United States of America), QIAzol (Qiagen, Germany), or GENEzol (Geneaid, Taiwan). The extraction of RNA with the TRIzol reagent has attracted much attention from researchers because of its universal properties and its relatively simple use. However, the standard protocol of this method is not always applicable to all types of cells, especially plant samples that have high fibre and polysaccharide content. Our tomato stem sample has a problem regarding the purity of A260/A280 and A260/A230 below 1.8. RNA extractions that were carried out on several samples such as soybean leaves, potato tubers, wheat seeds, and various medicinal plants with high secondary metabolites using the TRIzol method also did not provide maximum results (Behnam et al., 2019; Bilgin et al., 2009; Ghawana et al., 2011; Vennapusa et al., 2020). The low A260/A280 ratio is due to protein contamination.

In contrast, the low A260/A230 is caused by polysaccharides and residual phenol or guanidinium thiocyanate contamination from the reagent itself (Gallagher, 2017). Modification efforts to optimise the RNA recovery by adding polyvinylpyrrolidone (PVP) reagents (Deepa et al., 2014) and lithium chloride (LiCl) (Wang et al., 2009; Vennapusa et al., 2020) were carried out, but these approaches would add the production costs. Another alternative towards optimising RNA extraction that is often performed by researchers who encounter problems with RNA concentration and purity is adding or changing several mechanical steps. Repetition at the extraction stage using chloroform and washing RNA with ethanol increase RNA recovery both in quantity and quality (Roy et al., 2020; Toni et al., 2018; Vasanthaiah et al., 2008). Therefore, this study aimed was to improve the quantity and quality of RNA using the TRIzol optimisation method for obtaining high-quality RNA from tomato stem, thus providing valuable information for downstream assays.

METHODS

Sample Preparation

Commercial Thai tomato (*Solanum lycopersicum*) seeds are grown on soil mixture (humus, perlite, and vermiculite) in a greenhouse with a temperature of 24°C, regular sunlight (12 hours per day), and watered daily in the morning or evening depending on soil surface humidity. Tomato stems were collected after one month, cleaned with 70% alcohol beforehand and flash-frozen in liquid nitrogen before being stored in a -80°C freezer (Sanyo, Japan) to prevent cross-contamination and RNA degradation. The frozen stem samples were ground thoroughly with a mortar and pestle, previously heated at 180°C to remove enzymes that cause RNA degradation. The

grinding process is carried out by repeatedly mixing liquid nitrogen to prevent the sample from thawing.

RNA Extraction and Quality Assessment Using Spectrophotometry

RNA was extracted from the homogenised sample and was carried out following the TRIzol original method (Invitrogen, United States of America) based on the manufacturer's protocol and an optimised method with changes in the initial sample, i.e., 100 mg increased to 250 mg to 300 mg, twice chloroform extraction, and three times ethanol washing which are briefly described in Figure 1.

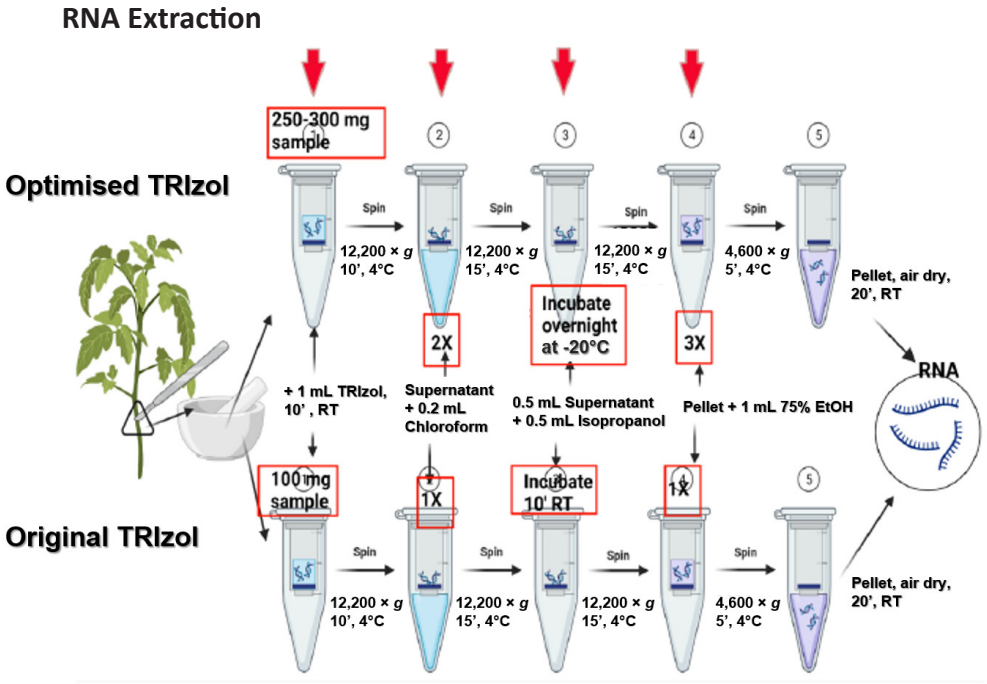

Figure 1. Workflow of RNA extraction using TRIzol reagent: Original protocol (lower part) and optimized protocol (upper part), the key step modification indicated in red arrow and the red square for each modification. Modifications including the initial amount of sample, twice chloroform extraction, overnight incubation in cold temperature, and three times ethanol wash

Optimised Method

An optimised used a 250 to 300 mg sample per 1 mL of the TRIzol reagent. The sample and reagent were put into a microtube, homogenised until dissolved and allowed to stand at room temperature for 10 minutes.

Next, the mixture was centrifuged at 4°C temperature and 12,200 × *g* for 10 minutes. The solution was divided into the aqueous phase (the supernatant) and organic phase. The supernatant was then transferred to a new microtube, 200 µL of chloroform was added, homogenised by inverting the microtube for 15 seconds, and allowed to stand for 3 minutes before being centrifuged at 4°C and 12,200 × *g* for another 15 minutes for phase separation. The supernatant was transferred into a new microtube for the second time and then re-extracted with 200 µL of chloroform to separate nucleic acids with proteins and organic compounds from the extraction reagents.

After two steps of chloroform extraction, the aqueous phase was transferred to a new microtube and mixed with cold isopropanol (1:1) for precipitation. The mixture was allowed to stand overnight at -20°C, then centrifuged at 4°C and 12,200 × *g* for 10 to 15 minutes to precipitate the pellets containing RNA. Next, the supernatant was discarded. Next, the RNA pellets were washed with 1 mL 75% ethanol [dissolved with diethylpyrocarbonate (DEPC) water], allowed to stand at room temperature, then centrifuged at 4,600 × *g* for 5 minutes. Agent, the supernatant was discarded. The washing step was repeated up to 3 times to maximise the washing of RNA from salt residues and organic compound residues.

RNA pellets were dried for 20 to 30 minutes at room temperature, dissolved in 20 µL of DEPC water, and then incubated at 55°C for 10 minutes using a heat block (Eppendorf, Germany) to dissolve. Concentration and purity were analysed using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, United States of America).

Formaldehyde Agarose Electrophoresis Gel

An electrophoresis gel run was performed to validate the presence of the extracted RNA. A 1.2% agarose gel was prepared by mixing 1.2 g of agarose, 10 mL of 10x MOPS (3-(N-morpholino)propanesulfonic acid) buffer (Sigma-Aldrich, United States of America), and 90 mL of DEPC water in Erlenmeyer flask (Pyrex®, United States of America). The MOPS buffer composition was adjusted according to Rio et al. (2015). The mixture was heated in the microwave for 1 to 2 minutes until dissolved, then cooled for 5 minutes before adding 1.8 mL of formaldehyde as a denaturing agent and 1 mL of ethidium bromide as a staining agent. The mixture was poured into the casting tray and let solidified before being immersed in 1 x MOPS buffer. RNA from each sample was adjusted to 500 ng with DEPC water up to 4 µL and then mixed with 4 µL 2 x RNA loading dye. The mixture was heated with a heat block at 70°C for 10 minutes and then chilled on ice before being loaded into a gel inside the electrophoresis machine (Analytik Jena, Germany). Electrophoresis was run on 55 V for 1 hour. The gel was visualised under a UV translluminator (Bio-Rad Laboratories, United States of America).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse transcriptase PCR was performed to validate the presence of mRNA for gene expression analysis. After sample quality was measured in spectrophotometry and electrophoresis analysis, elimination of genomic DNA from RNA sample and first-strand cDNA synthesis was carried out according to manufacturer's protocol (Thermo Fisher Scientific, United States of America). cDNA samples can be stored at -20°C for long-term storage or used directly for the polymerase chain reaction (PCR) technique. The primers used in this study are the ubiquitin (UBI3) gene with the sequence 5'- AGA AGA AGA CCT ACA CCA AGC C-3' (forward) and 5'-TCC CAA GGG TTG TCA CAT ACA -3' (reverse). UBI3 is a housekeeping gene produced by all cells in normal and stressful conditions (Løvdal & Lillo, 2009). The reverse transcriptase-polymerase chain reaction (RT-PCR) reaction was carried out with the following reaction mixture: 1 µL of cDNA template, 6 µL of nanopure water, 1 µL of enzyme KAPA SYBR® Fast Master Mix (Sigma-Aldrich, United States of America), and 2 µL of the UBI3 gene primer pair. The reaction was carried out in 30 cycles consisting of 3 minutes of pre-denaturation (95°C), 30 seconds of denaturation (94°C), 1 minute of annealing (60°C), and 1 minute of extension (72°C). The RT-PCR products were run in 1.5% agarose gel electrophoresis. The gel was prepared by mixing 0.75 g of agarose powder with 50 mL of 1x TBE (Tris-borate-EDTA) buffer (Sigma-Aldrich, United States of America) and then dissolved in the microwave for 1 minute before pouring it into the casting tray. Two (2) µL of DNA ladder (Thermo Fisher Scientific, United States of America) and cDNA samples were inserted into each well. Electrophoresis was run on 100 V for 40 minutes. The gel was then visualized using GelDoc UV transluminator (Bio-Rad Laboratories, United States of America).

RESULTS AND DISCUSSION

A pure RNA value of greater than 1.8 is universally accepted for A260/A280 and A260/A230 absorbance. Previously we tried to obtain RNA from tomato stems using the TRIzol method according to manufacturer protocol. However, the concentration and purity results were low. Therefore, we tried to optimise the method by changing mechanical steps as has been done in previous research with various samples (Behnam et al. 2019; Toni et al., 2018; Roy et al., 2020; Vasanthaiah et al., 2008). The data comparison of both yields is shown in Table 1.

Table 1

Concentration and purity of total RNA obtained from the stem of Solanum lycopersicum *with standard and optimized TRIzol protocols*

Protocol	RNA (µg/µL)	A260/A280	A260/A230
Original/Standard	0.35 ± 0.26	1.78 ± 0.18	0.9 ± 0.56
Optimized	0.76 ± 0.5	1.88 ± 0.09	2.07 ± 0.37

The use of 100 mg initial sample only produced an average of 0.35 µg/µL and low RNA quality by the electrophoresis analysis (Figure 3A). The addition of an initial sample of up to 300 mg was then used accordingly. It resulted in a higher concentration of 0.76 µg/µL (Figure 2A) and good RNA quality (Figure 3B). The sample to solvent ratio was previously made by Roy et al. (2020) on the adipose tissue sample. There is a correlation between the RNA recovery and the initial mass of a sample. RNA yield increased linearly with the addition in sample weight, meaning the increase in mass sample weight up to 300 mg per 1 mL TRIzol (3:10) affected the increase in RNA recovery. In addition, changes in precipitation conditions are also considered to affect the yield. Farrell Jr. (2017) recommended overnight precipitation for mature cell samples containing high secondary metabolites to ensure the recovery of RNA, which also has been done by Vasanthaiah et al. (2008) in grape tissue rich in polyphenols and polysaccharides. Li et al. (2020) also conducted comparisons of various duration and temperatures of nucleic acid precipitation. The results showed that the highest recovery rate occurred in microRNA (miRNA) samples (61%) and PCR products (33%) after overnight precipitation at -20°C.

The A260/A230 purity ratio from 9 samples extracted with original protocol which has value of 0.9 increased to 2.07 after optimization ($p = 0.001 < 0.05$, Figure 2C). Most of the samples extracted with the original protocol had a ratio of A260/A230 below 1.8, which indicated the presence of contamination such as polysaccharides, salts, and residual extraction reagents. This problem also occurred in previous studies using soybean leaf samples by Bilgin et al. (2009) and wheat seed samples by Vennapusa et al. (2020), which had A260/A230 ratios of 0.94 and 0.51, respectively. The results of the optimisation protocol in this study showed a significant improvement compared to the results of the standard protocol, which almost entirely did not meet the general standard A260/A230 ratio above 1.8. Therefore optimisation steps are highly recommended for RNA extraction from plant samples. Farrell Jr. (2017) stated that the repetition of chloroform extraction in the extraction stage for the second time could separate the remaining phenol from the aqueous phase in an equilibrium state after the first phase separation. A mixture of phenol and chloroform has also been shown to increase the yield of poly(A)$^+$ chain ends in mRNA, which has increased mRNA stability (Farrell Jr., 2017). For optimisation, the repetition has also been carried out in the final washing step, which removed extraction reagent residues (phenol and guanidinium thiocyanate) and most of the salts used to catalyse the deposition of nucleic acid molecules during precipitation.

The A260/A280 purity ratio of the optimised group gave a value of 1.88, slightly increased from the standard group (1.78) but statistically non-significant ($p = 0.309 > 0.05$, Figure 2B). It shows that the optimisation performed on optimised protocol does not have much effect in increasing the ratio. Previous research that

used the TRIzol method in plant samples by Bilgin et al. (2009) and Vennapusa et al. (2020) also produced an A260/A280 ratio in the range of 1.88 to 1.98, meaning that the TRIzol original protocol alone could produce an adequate A260/A280 ratio from plant samples rich of secondary metabolites. However, the optimisation protocol is suggested if the sample does not meet the value above 1.8.

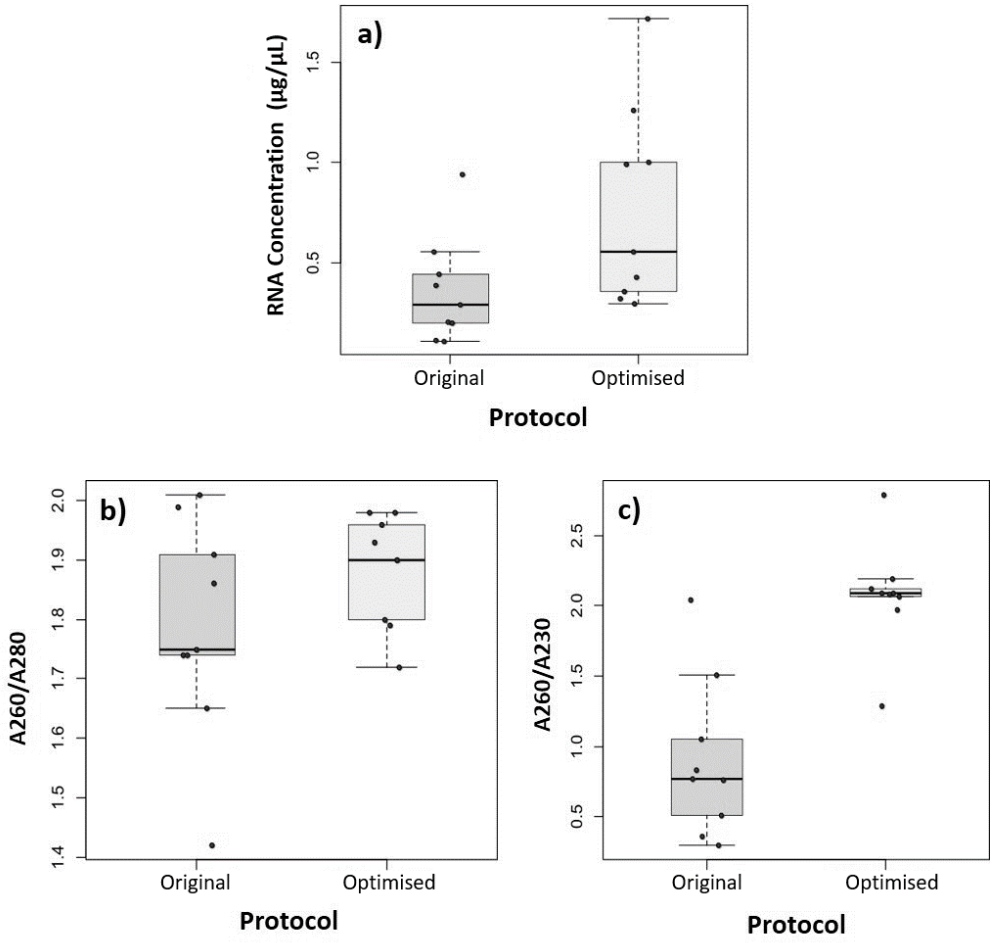

Figure 2. Box-and-Whisker Plot of RNA yield comparison from original and optimized protocols: (A) RNA concentration; (B) A260/A280 ratio; and (C) A260/A230 ratio (n = 9)

RNA integrity was then validated using the formaldehyde gel electrophoresis technique with standard TAE (Tris-acetate-EDTA)-based agarose gels (Sambrook & Russell, 2001) with three bands of different sizes as the indicator. Smear band and lack of 28S and 18S RNA intensity indicate low RNA quality from the samples extracted

with a standard protocol (Figure 3A). Similar results of electrophoresis band in plant RNA extracted by TRIzol, which has a purity of A260/A230 below 2.0, also occurred in *Dendrobium huosanense* sample (0.15-0.68) (Liu et al., 2018) and *Triticum aestivum* sample (0.51) (Vennapusa et al., 2020). In contrast, bright bands of RNA indicate the presence of good quality RNA, which could be seen in 8 of 9 samples from the optimised group (Figure 3B).

One of the samples with good quality both from spectrophotometry and electrophoresis (lane 2, Figure 3B) was used for further investigation in RT-PCR using the ubiquitin gene (UBI3) of the housekeeping genes that are always expressed in various conditions, including pathophysiological stress. The result showed that RNA samples were successfully converted into cDNA and used to analyse the expression of the gene, marked by a distinct 345 bp DNA band (Figure 4).

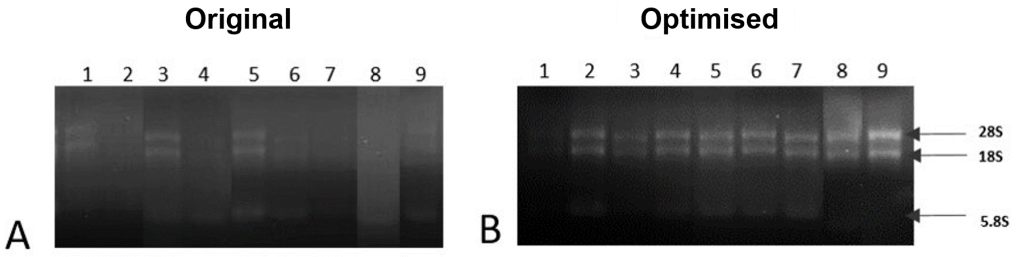

Figure 3. A 1.2% agarose electrophoresis gel of total RNA (9 samples) extracted using: (A) original TRIzol protocol and (B) optimized TRIzol protocol

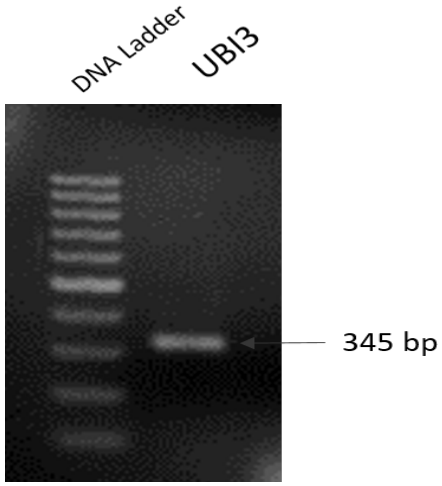

Figure 4. RT-PCR profile from *Solanum lycopersicum* housekeeping gene ubiquitin (UBI3) primer using RNA as template from optimized protocol

CONCLUSION

This optimised TRIzol-based protocol was proven to yield a higher quantity and quality of extracted RNA from tomato stems. The optimisation steps include adding the initial sample amount up to three times, twice chloroform extraction, overnight incubation in low temperature (-20°C), and three times ethanol washing. The improvement in RNA quality was seen from the A260/A230 ratio, which significantly increased, indicating the treatments successfully purified RNA from contamination of organic residues such as salt and phenol. Improvement of the A260/A230 ratio could improve RNA, proven by the entire electrophoresis band of 8 from 9 RNA samples (89%) in the optimised group. RNA with good concentration and purity from the optimised group was used for reverse transcriptase-polymerase chain reaction (RT-PCR) and resulted in the amplicon band of ubiquitin gene.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the author(s).

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Effects of Cutting Process and Drying Period using Sunlight on Hay Quality of Dwarf Napier Grass (*Pennisetum purpureum*) and *Asystasia gangetica*

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ABSTRACT

This study aims to determine the effect of the cutting process and drying period using sunlight on the hay quality of dwarf Napier grass (*Pennisetum purpureum*) and *Asystasia gangetica*. Hay quality of both species was evaluated in a completely randomised design corresponding to five drying periods (one to five days), with or without cutting process, with three replicates. Both plants were harvested at a vegetative stage and then divided into two portions: unchopped and chopped. Plants were dried using sunlight for the respective drying period, and hay quality was examined in physical and chemical analyses. Each sample was analysed for dry matter (DM) content. The *A. gangetica* at four days drying period and Napier grass at five days drying period were selected for chemical analysis. Results showed that the physical characteristics of hays for both plants were not affected by the drying periods and cutting process. *Asystasia gangetica* achieved higher DM content than Napier grass for almost all drying periods. For Napier grass, the three days were drying periods that achieved the desirable DM content (> 85.0%), while two days were drying periods for *A. gangetica*. Napier grass contained higher crude fibre and ether extract contents than *A. gangetica*, while crude protein content appeared *vice versa*. The nutritive values of both plants were not affected by the cutting process. In conclusion, Napier grass's three days drying

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periods and a two days drying period of *A. gangetica* can achieve the desirable DM content under sunny conditions.

Keywords: *Asystasia gangetica*, drying period, dwarf Napier grass, hay quality, *Pennisetum purpureum*, sunlight

INTRODUCTION

There are many tropical and sub-tropical forage grasses in the country, some examples being Napier grass (*Penniseutm puprpureum*), Guinea grass (*Panicum maximum*), and setaria (*Setaria sphacelata*). Therefore, it is possible to get a surplus amount of biomass during the rainy season, which farmers cannot finish by supplying to their animals. Moreover, farmers cannot keep the grasses in their fields for a long time because the nutritive quality of plants deteriorates with the advancement of maturity (Shewfelt, 1990). In this circumstance, it is better to preserve their surplus production as silage or hay. Moreover, by preserving the surplus amount of grass, farmers can use the preserved grass for feeding their animals during dry periods or feed scarcity (Tripathi, 1995).

Napier grass is gaining popularity among farmers among the forage grasses because of its high dry matter (DM) yield, moderate nutritive value, ease of propagation, drought tolerance, and low management. Napier grass has two main varieties: tall and dwarf. Halim et al. (2013) reported that tall Napier grass varieties showed higher DM yield and lower nutritive value than dwarf varieties. Due to the lower leaf-stem ratio in tall varieties compared to dwarf varieties, farmers preserve them as silage. Therefore, it is not considered to preserve it by making hay. However, dwarf varieties of Napier grass show a high leaf-stem ratio and may be preserved as hay.

Since there is a longer duration of sunlight in Malaysia, it is possible to make hay using natural sunlight. However, for silage it takes more time, labour, and equipment than haymaking. Hay quality can significantly differ even within one species grown in the same locality. The variation occurs primarily because of an absence of understanding of good haymaking fundamentals and the tendency of farmers to offer less attention to hay crops than to soybeans, corn, small grains, and other crops. Farmers can cut their feed-spending cost by making their hay with good-quality hay rather than buy poor-quality hay from the supplier. Higher quality hay can provide essential nutrients that forage cannot supply because it contains high nutritive content, including crude protein (CP) and digestible energy. The objective of this study was to investigate the effects of 'cutting into pieces' and 'drying period using sunlight' on physical characteristics and nutritive quality of dwarf Napier grass and *A. gangetica* hay.

MATERIALS AND METHODS

Plant Materials and Sample Preparation

Dwarf Napier grass at about two months of plant maturity was collected from Susu Kambing Mache Klate, Machang, Kelantan. In contrast, *Asystasia gangetica* was collected at the vegetative stage from Agro Techno Park, Universiti Malaysia Kelantan, Jeli Campus, Kelantan. About half of the grasses for each species of plants were cut manually into two, termed as chopped. The remaining grasses for each species of plant were termed as unchopped. Dwarf Napier grass and *A. gangetica* were evaluated for hay quality. Treatments consisted of five drying periods: one day, two days, three days, four days, and five days. Each drying period was evaluated with unchopped and chopped plant materials. A completely randomised design with three replications was adopted. Based on a good weather forecast, about 1.0 kg grass for each replication was dried on a plastic sheet (1 m × 1 m) under the sun for respective treatment. The drying period consisted of eight hours in one day, from 9:00 a.m. to 5:00 p.m. The grass was turned over at 1:00 p.m. daily to speed up the drying process. The maximum, minimum and average temperatures during October 2020 were 30.6°C, 24.0°C, and 27.3°C, respectively, while the average rainfall and humidity were 323 mm and 90%, respectively.

Physical and Chemical Analyses

Physical analysis was performed by evaluating the hays of dwarf Napier grass and *A. gangetica* on the following: stage of maturity, leafiness, colour, odour and conditions, and foreign materials, as shown in Table 1. The percentage of leaves (old and brown) was observed by visual inspection, and the score was given (Table 1). Samples of all hays were analysed for DM following the Association of Official Analytical Chemists (AOAC) (2000). However, samples of *A. gangetica* at four days drying period and Napier grass at five days drying period were analysed for determination of CP, crude fibre (CF), ether extract (EE), and ash contents following the method of AOAC (2000).

Table 1

Standard scores for measuring the physical quality of hay (Vough, 2000)

	Characteristics	Score
Maturity		
i)	0-5% of leaves are old and brown	27-30
ii)	6-15% of leaves are old and brown	22-26
iii)	16-30% of leaves are old and brown	17-21
iv)	>30% of leaves are old and brown	11-16

Table 1 *(Continued)*

	Characteristics	Score
Leafiness		
i)	Very leafy	17-20
ii)	Leafy	12-16
iii)	Few leaves	7-11
iv)	Not leafy	1-6
Colour		
i)	The bright green colour of the crop	15-20
ii)	Golden yellow to yellow hays 5-15	5-15
iii)	Brown or black	0-5
Odour and conditions		
i)	The smell of new-mown hay	15-20
ii)	Musty or off-odours	5-15
iii)	Dusty	0-5
Foreign materials		
Hay with non-harmful foreign material should receive a lower score than that without. Hay with harmful foreign material should not be fed to animals.		1-10

Statistical Analysis

The results for different drying periods were subjected to analysis of variance. Differences among means were tested using Duncan Multiple Range Test (DMRT) or *F* test, with significance at $p<0.05$, by Statistical Package for the Social Sciences (SPSS) software (Version 22.0). In addition, the student's *t*-test was used to compare the means between two groups (Napier grass *vs A. gangetica* and unchopped *vs* chopped) at $p<0.05$.

RESULTS AND DISCUSSION

Physical Characteristics

Maturity was observed at the time when the grass was being harvested. Table 2 shows that scores for maturity were above 22 among all the treatments, which indicated that 6% to 15% of leaves from the collected samples were old and brown. Leafiness plays a critical role in getting high-quality hay because more vitamins and minerals can be found in the leaves. As shown in Table 2, the leafiness score for each treatment was more than 11.0%, which indicated that both experimental plants represented leafy characters. Leafiness percentage can be lost due to improper handling and leaves becoming too dry (thus causing them to fall off from the stem). The low percentage of leafiness in hay reduces feed value (Vough, 2000).

Table 2

Scores (mean ± standard deviation) for physical characteristics for dwarf Napier grass and Asystasia gangetica *hays regardless of drying periods*

Characteristics	Dwarf Napier grass		*Asystasia gangetica*		*p*-value
	Unchopped	Chopped	Unchopped	Chopped	
Maturity	24.2 ± 0.84	25 ± 1.0	23.8 ± 0.45	24.8 ± 0.84	0.113
Leafiness	16.0 ± 1.87	15.4 ± 2.07	16.2 ± 2.05	15.2 ± 2.05	0.839
Colour	16.6 ± 1.82	16.2 ± 2.39	16.6 ± 1.82	16.6 ± 2.41	0.987
Odour and conditions	16.0 ± 1.87	16.8 ± 1.92	16.2 ± 1.92	16.8 ± 1.92	0.872
Foreign materials	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00	1.000

The most favourable colour in haymaking is bright green. However, the colour of hay is not the decisive factor in deciding the quality of hay because it cannot honestly decide its nutritive value (Rocateli & Zhang, 2017). Slight discolourations from sun bleaching, dew, or moderate fermentation are not as severe as the loss of green colour from maturity, rain damage, and excessive fermentation or heating. Colour was not significantly different (p>0.05) among the treatments (Table 2). The smell of hay plays a vital role in acceptance as feed by animals since the smell acts as an appetiser before eating. The smell of new-mown hay indicates high-quality hay, while hay emitting off-odours indicates low-quality hay (Rocateli & Zhang, 2017). There were no differences in odour scores among the treatments. Foreign materials can be easily observed in hay because the different materials from the hay are apparent (Rocateli & Zhang, 2017; Vough, 2000). There are two types of foreign materials: injurious and non-injurious materials. Non-injurious materials are usually not harmful to animals because they are usually waste from the surroundings attached to the hay. Meanwhile, harmful materials are dangerous to animals because they can injure them and, in the worst case, cause death. Based on Table 2, the scores for the foreign materials were all ten among the treatments, which indicate that there were no foreign materials in the hay of this study. This condition is probably caused because the study used a low quantity of hay.

Chemical Composition

Dry Matter. Dry matter content is crucial in haymaking. It is an essential factor in the long-term preservation of hay, which avoids the undesirable growth of fungus and mould. Typically, hay should contain at least 85% DM (Lemus, 2020). Table 3 shows that the DM contents of Napier grass and *A. gangetica* were significantly (p<0.05)

affected by drying periods irrespective of whether they are chopped or not. *Asystasia gangetica* achieved significantly higher DM content than dwarf Napier grass for each drying period (except at day three). The highest DM value of Napier grass (87.3%) was recorded on day five, while the lowest DM value (67.7%) was recorded on day one. Although Napier grass hay on days two and three did not achieve the desired DM content, Napier grass hay achieved the desired DM content on days three, four, and five, which was 86.5% or more. Results indicated that it is possible to make hay from dwarf Napier grass within three days, whether chopped or not if dwarf Napier grass is dried using the scorching heat usually found in Malaysia. The DM contents of dwarf Napier grass hay that was dried for three to five days are in line with the findings of Mapato and Wanapat (2018). They reported that dwarf Napier grass contained 85.7% DM that was dried for three to five days by sun-drying.

Table 3

Differences of dry matter content (%) (mean ± standard deviation) in dwarf Napier grass and Asystasia gangetica *hays (irrespective of whether they are chopped or not)*

Drying period	Dwarf Napier grass	*Asystasia gangetica*	*p*-value
One day	67.7 ± 11.3^{aA}	79.8 ± 3.5^{bA}	0.015
Two days	82.4 ± 3.1^{aB}	92.5 ± 2.0^{bC}	0.000
Three days	87.2 ± 1.9^{B}	86.4 ± 0.8^{B}	0.157
Four days	86.5 ± 1.3^{aB}	91.1 ± 0.3^{bC}	0.000
Five days	87.3 ± 1.3^{aB}	83.9 ± 1.5^{bB}	0.001
Overall	82.2 ± 9.1^{a}	86.7 ± 5.1^{b}	0.010
p-value	0.000	0.000	

Note. Means within rows followed by different lower case letters and within columns followed by different upper case letters differ ($p<0.05$)

As shown in Table 4 (irrespective of the species), the DM content was not affected by each drying period between unchopped and chopped hays. As expected, for both unchopped and chopped hays, the DM content of hay at a one day drying period was significantly lower DM than the other drying periods. However, the DM values were not affected by each drying period (except at two days) between unchopped and chopped hays of Napier grass (Table 5). At two days drying period, chopped hay achieved significantly ($p<0.05$) lower DM content (79.6 vs 85.3%) than the unchopped hay, respectively. It is also shown that unchopped hay of Napier grass achieved the desired DM content (85% or more) at a minimum of two days drying period. In contrast, it was achieved at three days drying period for chopped hay of Napier grass.

Table 4

Differences of dry matter content (%) (mean ± standard deviation) in unchopped and chopped hays (irrespective of the species)

Drying period	Unchopped	Chopped	*p*-value
One day	70.3 ± 13.5^{A}	77.2 ± 4.1^{A}	0.128
Two days	89.1 ± 4.3^{B}	85.8 ± 7.0^{B}	0.175
Three days	87.0 ± 1.0^{B}	86.6 ± 1.9^{B}	0.335
Four days	88.8 ± 2.3^{B}	88.7 ± 3.0^{B}	0.474
Five days	85.5 ± 3.0^{B}	85.7 ± 1.4^{B}	0.456
Overall	84.1 ± 9.4	84.8 ± 5.5	0.371
p-value	0.000	0.001	

Note. Means within columns followed by different upper case letters differ ($p<0.05$)

Table 5

Differences of dry matter content (%) (mean ± standard deviation) in unchopped and chopped hays of dwarf Napier grass

Drying period	Unchopped	Chopped	*p*-value
One day	61.1 ± 13.6^{A}	74.3 ± 1.7^{A}	0.085
Two days	85.3 ± 0.9^{bB}	79.6 ± 0.3^{aB}	0.000
Three days	87.7 ± 0.6^{B}	86.8 ± 2.9^{C}	0.317
Four days	86.8 ± 1.1^{B}	86.1 ± 1.6^{C}	0.270
Five days	87.7 ± 1.6^{B}	86.8 ± 0.9^{C}	0.216
Overall	81.7 ± 11.9	82.7 ± 5.4	0.385
p-value	0.001	0.000	

Note. Means within rows followed by different lower case letters and within columns followed by different upper case letters differ ($p<0.05$)

Asystasia gangetica hay achieved the desired DM content on day two, which was more than 85.0%. The highest DM value (92.5%) for *A. gangetica* hay was recorded on day two. In comparison, the lowest DM value (79.8%) was recorded on day one. Meanwhile, the DM values were not affected by the drying periods between unchopped and chopped hays of *A. gangetica* (except on day four) (Table 6). On day four, chopped hay achieved higher DM content (91.3 *vs* 90.8%) than the unchopped hay, respectively. Sobayo et al. (2012) reported that chopped *A. gangetica* sun-dried DM value was 85.5%. However, the drying period was not stated in the Sobayo et al. (2012) study. Therefore, the data in Tables 3, 4, 5, and 6 agree with the findings

of Sobayo et al. (2012) because the DM contents in both hays of this current study were more than 85% when it was sun-dried for three days or more.

The temperature during the drying process of this study in October was a suitable temperature to make hay. In addition, the percentage of humidity was relatively high in that month (90.0%). The air moving across the top of the drying hay crop must absorb the water that is evaporating and mix it with the rest of the atmosphere. In this regard, air behaves much like a sponge or a mop (Evans, 1975). The humidity is high, so the hay will absorb the water from the air and increase its moisture. In October, the average wind speed recorded was 4.18 mph (miles per hour) which is deemed relatively low. Since most drying takes place during daylight hours, wind speed is an essential factor. If the wind speed is low, the air next to the crop surface will soon become saturated and be unable to absorb any water.

Table 6

Differences of dry matter content (%) (mean ± standard deviation) in unchopped and chopped hays of Asystasia gangetica

Drying period	Unchopped	Chopped	*p*-value
One day	79.5 ± 4.1^{A}	80.1 ± 3.7^{A}	0.426
Two days	93.0 ± 0.7^{C}	92.0 ± 3.0^{C}	0.312
Three days	86.3 ± 1.0^{B}	86.4 ± 0.7^{B}	0.445
Four days	90.8 ± 0.2^{aC}	91.3 ± 0.0^{bC}	0.005
Five days	83.3 ± 2.1^{B}	84.5 ± 0.6^{B}	0.188
Overall	86.6 ± 5.4	86.9 ± 4.9	0.434
p-value	0.000	0.000	

Note. Means within rows followed by different lower case letters and within columns followed by different upper case letters differ ($p<0.05$)

Nutritive Value. As shown in Table 7 (whether they are chopped or not), the CP content was higher in *A. gangetica* than in Napier grass. In contrast, the CF and EE contents appeared *vice versa*. However, there was no significant difference in ash content between Napier grass and *A. gangetica*. This difference might be attributed due to the use of different species.

Irrespective of the species, there were no differences ($p<0.05$) on the CP, CF, EE, and ash contents between unchopped and chopped hays (Table 8). The Napier grass's proximate components (except for CF) were not affected by the cutting process (Table 9). The CP, CF, EE, and ash contents of unchopped Napier grass were 15.4%, 22.0%, 0.6%, and 15.7%, while the contents

for chopped Napier grass were 15.0%, 24.4%, 0.8%, and 14.6%, respectively. Similarly, the proximate components (except for EE) of *A. gangetica* were not affected by the cutting process. The CP, CF, EE, and ash contents of unchopped *A. gangetica* were 18.1%, 17.6%, 0.1%, and 13.9%. In comparison the contents for chopped *A. gangetica* were 17.6%, 20.8%, 0.3%, and 11.1%, respectively. The above findings indicated that the cutting process might not influence the proximate components. Like the current study, Mapato and Wanapat (2018) reported that dwarf Napier grass contained 15.1% CP. In another study, Sobayo et al. (2012) reported that the CP content of *A. gangetica* leaf meal was 19.38%, slightly higher than the current study. The CF value in this study is within the range of the reported value by Rahman et al. (2020). From the study of Sobayo et al. (2012), the CF content for *A. gangetica* leaf meal was 15.3%, slightly lower than the recorded value in this study. The EE value of dwarf Napier grass in this study was lower than the findings of Rahman et al. (2020); it may have occurred due to the use of plants with different maturities. Maturity is one of the crucial factors in determining hay quality. Sobayo et al. (2012) found 12.7% EE in *A. gangetica*, significantly higher than the EE value found in this study. In the Rahman et al. (2020) study, the reported ash value was slightly lower (10.2%) than the ash content found in this study. In contrast, the ash content recorded in the Sobayo et al. (2012) study was much lower (1.74%) than what was recorded in this study.

Table 7

Composition of proximate components (%) (mean ± standard deviation) in dwarf Napier grass and Asystasia gangetica *hays (irrespective of whether they are chopped or not)*

Parameter	Dwarf Napier grass (sun-drying for 5 days)	*Asystasia gangetica* (sun-drying for 4 days)	*p*-value
Crude protein (%)	15.2 ± 2.0^{a}	17.9 ± 1.9^{b}	0.018
Crude fibre (%)	23.2 ± 1.7^{b}	19.2 ± 3.0^{a}	0.009
Ether extract (%)	0.7 ± 0.2^{b}	0.2 ± 0.1^{a}	0.000
Ash (%)	15.1 ± 2.4	12.5 ± 4.1	0.100

Note. Means within rows followed by different lower case letters differ ($p<0.05$)

Table 8

Composition of proximate components (%) (mean ± standard deviation) in unchopped and chopped hays (irrespective of the species)

Parameter	Unchopped	Chopped	*p*-value
Crude protein (%)	16.8 ± 3.0	16.3 ± 1.5	0.376
Crude fibre (%)	19.8 ± 3.2	22.6 ± 2.6	0.061
Ether extract (%)	0.4 ± 0.3	0.5 ± 0.3	0.152
Ash (%)	14.8 ± 3.1	12.8 ± 3.9	0.178

Table 9

Composition of proximate components (%) (mean ± standard deviation) in unchopped and chopped hays within each species

Species	Parameter	Unchopped	Chopped	*p*-value
Dwarf Napier grass (sun-drying for 5 days)	Crude protein (%)	15.4 ± 3.0	15.0 ± 0.7	0.423
	Crude fibre (%)	22.0 ± 1.1[a]	24.4 ± 1.4[b]	0.037
	Ether extract (%)	0.6 ± 0.2	0.8 ± 0.2	0.117
	Ash (%)	15.7 ± 3.3	14.6 ± 1.7	0.312
Asystasia gangetica (sun-drying for 4 days)	Crude protein (%)	18.1 ± 2.9	17.6 ± 0.4	0.381
	Crude fibre (%)	17.6 ± 3.1	20.8 ± 2.4	0.112
	Ether extract (%)	0.1 ± 0.0[a]	0.3 ± 0.1[b]	0.024
	Ash (%)	13.9 ± 3.2	11.1 ± 5.2	0.236

Note. Means within rows followed by different lower case letters differ ($p<0.05$)

CONCLUSION

Physical analysis of dwarf Napier grass and *Asystasia gangetica* hays was not significantly affected by drying and cutting. For dwarf Napier grass, the three-days drying period achieved the desirable DM content (>85.0%). In contrast, for *A. gangetica*, the two-days drying period achieved the desirable DM content. The nutritive values of dwarf Napier grass and *A. gangetica* were not affected by the cutting process. The CF and EE contents were higher in dwarf Napier grass than *A. gangetica*, while the CP content appeared *vice versa*.

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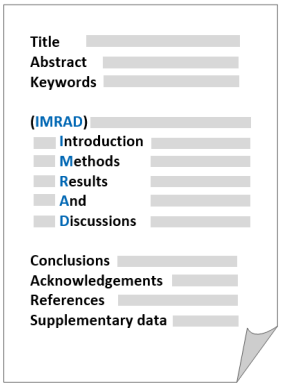

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	Insertion in text	**In reference list**
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Book/E-Book with 3 or more authors	*For all in-text references, list only the first author's family name and followed by* 'et al.' **Information prominent' (the author's name is within parentheses):** … (Karam et al., 2017) … Or **'Author prominent' (the author's name is outside the parentheses):** Karam et al. (2017) …	Karam, D. S., Abdu, A., Rajoo, K. S., Jamaluddin, A. S., & Karim, R. (2017). *Tropical forest soil characteristics in rehabilitated forests of Malaysia*. UPM Press.
Book/E-Book with more than 20 authors		For books with more than 20 authors, please follow the guidelines for journal articles with more than 20 authors.
Chapter in an edited Book/E-Book	**Information prominent' (the author 's name is within parentheses):** … (Kaur, 2020) … … (Tautges & Seow, 2020) … Or **'Author prominent' (the author's name is outside the parentheses):** Kaur (2020) … Tautges and Seow (2020) …	Kaur, G. (2020). Microbial phytases in plant minerals acquisition. In V. Sharma, R. Salwan, & L. K. T. Al-Ani (Eds.), *Molecular aspects of plant beneficial microbes in agriculture* (pp. 185-194). Academic Press. https://doi.org/10.1016/B978-0-12-818469-1.00016-X Tautges, N., & Seow, K. (2020). Pursuing agroecosystem in a long-term Mediterranean agricultural experiment. In G. Bhullar & A. Riar (Eds.), *Long-term farming systems research: Ensuring food security in changing scenarios* (pp. 53-66). Academic Press. https://doi.org/10.1016/B978-0-12-818186-7.00004-7

	Insertion in text	**In reference list**
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Several works by the same author in the same year	**Information prominent' (the author's name is within parentheses):** … (Arya et al., 2020a, 2020b) … Or **'Author prominent'(the author's name is outside the parentheses):** Arya et al. (2020a, 2020b) …	Arya, R. L., Arya, S., Arya, R., & Kumar, J. (2020a). Genetics, plant breeding and biotechnology. In *Fundamentals of agriculture: General agriculture - Agronomy* (Vol. 1, pp. 71-143). Scientific Publishers. Arya, R. L., Arya, S., Arya, R., & Kumar, J. (2020b). Seed science. In *Fundamentals of agriculture: General agriculture - Agronomy* (Vol. 1, pp. 196-215). Scientific Publishers.
	Journals	
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
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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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	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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