MORPHOMETRIC ANALYSIS AND GENETIC VARIATION OF SELECTED BORNEAN STINGLESS BEE, *TETRAGONULA* SPP. (HYMENOPTERA: MELIPONINI) AND ITS POTENTIAL AS POLLINATORS FOR *VANILLA PLANIFOLIA* AND *HYLOCEREUS POLYRHIZUS*

KIMBERLY ANAK ADOR

FACULTY OF SUSTAINABLE AGRICULTURE
UNIVERSITI MALAYSIA SABAH
2023

MORPHOMETRIC ANALYSIS AND GENETIC VARIATION OF SELECTED BORNEAN STINGLESS BEE, *TETRAGONULA* SPP. (HYMENOPTERA: MELIPONINI) AND ITS POTENTIAL AS POLLINATORS FOR *VANILLA PLANIFOLIA* AND *HYLOCEREUS POLYRHIZUS*

KIMBERLY ANAK ADOR

THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN AGRICULTURAL SCIENCE

FACULTY OF SUSTAINABLE AGRICULTURE
UNIVERSITI MALAYSIA SABAH
2023

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS

JUDUL : **MORPHOMETRIC ANALYSIS AND GENETIC VARIATION OF SELECTED BORNEAN STINGLESS BEE, *TETRAGONULA* SPP. (HYMENOPTERA: MELIPONINI) AND ITS POTENTIAL AS POLLINATORS FOR *VANILLA PLANIFOLIA* AND *HYLOCEREUS POLYRHIZUS***

IJAZAH : **DOKTOR FALSAFAH SAINS PERTANIAN**

BIDANG : **HORTIKULTUR**

Saya **KIMBERLY ANAK ADOR**, Sesi **2017-2023**, mengaku membenarkan tesis Doktoral ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:

1. Tesis ini adalah hak milik Universiti Malaysia Sabah
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat Salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. Sila tandakan (/):

[ ]	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA 1972)
[ ]	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan
[/]	TIDAK TERHAD	

KIMBERLY ANAK ADOR
DR1711003T

Tarikh : 31 Mac 2023

Disahkan Oleh,
ANITA BINTI ARSAD
PUSTAKAWAN KANAN
UNIVERSITI MALAYSIA SABAH
(Tandatangan Pustakawan)

Prof. Madya Dr. Suzan@Sarah Benedick
Pensyarah
Fakulti Pertanian Lestari, UMS Sandakan

(Prof. Madya Dr. Suzan Benedick)
Penyelia Utama

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, equations, summaries and references, which have been fully acknowledged.

1 July 2022

.....
Kimberly Anak Ador
DR1711003T

CERTIFICATION

NAME	:	**KIMBERLY ANAK ADOR**
MATRIC NO.	:	**DR1711003T**
TITLE	:	**MORPHOMETRIC ANALYSIS AND GENETIC VARIATION OF SELECTED BORNEAN STINGLESS BEE, *TETRAGONULA* SPP. (HYMENOPTERA: MELIPONINI) AND ITS POTENTIAL AS POLLINATORS FOR *VANILLA PLANIFOLIA* AND *HYLOCEREUS POLYRHIZUS***
DEGREE	:	**DOCTOR OF PHILOSOPHY IN AGRICULTURAL SCIENCE**
FIELD	:	**HORTICULTURE**
VIVA DATE	:	**1 JULY 2022**

CERTIFIED BY;

Signature

1. MAIN SUPERVISOR
Assoc. Prof. Dr. Suzan Benedick@Sarah Abdullah

Prof. Madya Dr. Suzan@Sarah Benedick
Pensyarah
Fakulti Pertanian Lestari, UMS Sandakan

2. CO-SUPERVISOR
Ts. Dr. Januarius Gobilik

TS. DR. JANUARIUS GOBILIK
SENIOR LECTURER
FACULTY OF SUSTAINABLE AGRICULTURE
UNIVERSITI MALAYSIA SABAH

ACKNOWLEDGEMENT

I would like to thank Associate Professor Dr. Suzan Benedick for her moral supports, ideas, comments, finance and time. Without her kind guidance, I would not be able to finish my doctorate degree study successfully. I would like to express my gratitude to Ts. Dr. Januarius Gobilik, my co-supervisor, for his comments and suggestions to improve my thesis writing. I also would like to thank all lecturers in Faculty of Sustainable Agriculture for all their comments and all the laboratory staff who had helped me throughout my study.

I also would like to extend my gratitude to all my family members and friends for their supports and prayers. Special thanks to my husband (Yaltoh), parents (Mr. Ador and Mdm. Sue), my brother (Dawson) and sisters (Sarah and Joan) who encourage me to pursue my doctorate degree, and for all their prayers and moral supports. I am also indebted to my friends for helping me throughout my research and their moral supports.

Last but not least, million thanks to those who had helped me directly and indirectly throughout my doctorate study.

Kimberly Anak Ador
1 July 2022

ABSTRACT

This study is mainly concerned with one of the most abundant stingless bees with many cryptic species in Malaysia, *Tetragonula* spp. (Hymenoptera; Meliponini). The aims of this study are i) to determine the habitat preferences, morphometric and morphological traits, and phylogenetic relationships of selected species of cryptic Bornean *Tetragonula*, ii) to investigate the morphological and molecular characters of cryptic speciation in *Tetragonula laeviceps*, iii) to investigate the potential of *T. laeviceps* as a pollinator of vanilla and pitaya flowers, and iv) to investigate the antioxidant activity, phenolic compound and chemical composition in products of the stingless bee. This study was conducted from October 2017 to February 2021. Samples of workers of the genus *Tetragonula* were collected from the Malaysian states of Sabah (Tuaran, Putatan, Kinarut, Kota Marudu, Kudat, Beaufort, Beluran and Sandakan, Sabah) and Sarawak (Tebedu) to study their morphological characteristics, molecular analysis and quality of bee products. DNA was extracted from the whole insect, amplified by polymerase chain reaction (PCR) and sequenced to obtain suitable data for molecular analysis. The pollination efficiency of *T. laeviceps* on pitaya (*Hylocereus polyrhizus*) and vanilla (*Vanilla planifolia*) was studied at the Faculty of Sustainable Agriculture. Honey, pollen and propolis from *T. laeviceps* and *Heterotrigona itama* were used to analyse antioxidants and compare the quality of stingless bee products. Five species of *Tetragonula* were examined in this study, namely *T. laeviceps*, *T. melanocephala*, *T. fuscobalteata*, *T. iridipennis* and *T. ruficornis*. Pearson's correlation showed that there was a significant difference between the morphometric traits and factor 1 score analysis of *Tetragonula* spp. ($p < 0.05$). The use of both morphometric characters and phylogenetic classification to distinguish cryptic species of stingless bees could increase the accuracy of identification ($p < 0.001$). The most important morphological characters of *T. laeviceps* to distinguish between groups or subspecies were body size and body colouration. The relatively large genetic or intraspecific distance within the phylogenetic relationships of *T. laeviceps*, resulting in four different clades, suggests the possibility of cryptic species. Pollination by *T. laeviceps* resulted in better fruit quality in terms of fruit weight, fruit length and fruit diameter of pitaya, while vanilla was completely dependent on hand pollination. There were significant differences between the pollen samples of *T. laeviceps* and *H. itama* in terms of antioxidant activity as measured by t-test ($p < 0.05$). There were no significant differences in pH analysis in all samples of honey, pollen and propolis from *T. laeviceps* and *H. itama*, measured with the t-test ($p > 0.05$). Twenty-eight chemical compounds were identified in the honey samples of *T. laeviceps* and *H. itama*. Amino acids, gamma-butyrolactones and steroidal saponins were identified as major constituents as they were present in all honey samples.

Keywords: Morphometric variation, genetic variation, phylogenetic relationships, stingless bee, Tetragonula spp., pollination

ABSTRAK

ANALISIS MORFOMETRIC DAN VARIASI GENETIK KELULUT BORNEO YANG TERPILIH, TETRAGONULA SPP. (HYMENOPTERA: MELIPONINI) DAN POTENSINYA SEBAGAI PENDEBUNGA KEPADA VANILLA PLANIFOLIA DAN HYLOCEREUS POLYRHIZUS

Kajian ini mengutamakan salah satu spesies kelulut yang paling banyak dan mempunyai banyak species krip di Malaysia, Tetragonula spp. (Hymenoptera: Meliponini). Sasaran kajian ini termasuklah i) untuk menentukan pemilihan habitat, sifat morfometrik dan morfologi, dan hubung kait filogenetik antara spesies krip Tetragonula Borneo, ii) untuk mengkaji karakter morfologi dan molekular spesiasi krip pada Tetragonula laeviceps, iii) untuk mengkaji potensi T. laeviceps sebagai pendebunga kepada bunga vanilla dan pitaya, dan iv) untuk mengkaji aktiviti antioksidan, sebatian fenolik dan komposisi kimia dalam produk kelulut. Kajian ini dijalankan dari Oktober 2017 hingga Februari 2021. Sampel pekerja genus Tetragonula diambil daripada negeri di Malaysia iaitu Sabah (Tuaran, Putatan, Kinarut, Kota Marudu, Kudat, Beaufort, Beluran dan Sandakan) dan Sarawak (Tebedu) untuk kajian ciri-ciri morfologi, analisis molekul dan kualiti produk lebah. DNA diekstrak daripada seluruh badan serangga, diasingkan dengan polymerase chain reaction (PCR) dan dijujukkan bagi mendapatkan data yang sesuai untuk analisis molekul. Kecekapan pendebungaan oleh T. laeviceps terhadap pitaya (Hylocereus polyrhizus) dan vanilla (Vanilla planifolia) telah dikaji di Insektarium, Fakulti Pertanian Lestari, Universiti Malaysia Sabah. Madu, roti debunga dan propolis dari T. laeviceps dan Heterotrigona itama digunakan untuk analisis antioksidan dan perbandingan kualiti produk kelulut. Lima spesies Tetragonula dalam kajian ini telah dikenal pasti iaitu T. laeviceps, T. melanocephala, T. fuscobalteata, T. iridipennis dan T. ruficornis. Korelasi Pearson's menunjukkan perbezaan yang ketara antara sifat morfometrik dan skor faktor 1 Tetragonula spp. ($p < 0.05$). Pengunaan kedua-dua ciri morfometrik dan pengelasan filogenetik untuk membezakan spesies krip dapat meningkatkan ketepatan dalam pengenalpastian ($p < 0.001$). Karakter morfologi T. laeviceps yang paling penting untuk membezakan antara kumpulan dan subspecies ialah saiz badan dan warna badan. Genetik atau jarak intraspesifik yang besar di dalam hubungan filogenetik T. laeviceps menghasilkan 4 klad yang berbeza, menunjukkan terdapat spesies krip. Pendebungaan oleh T. laeviceps menghasilkan buah yang lebih berkualiti dari segi berat, lebar dan diameter buah pitaya, manakala vanilla bergantung sepenuhnya kepada pendebungaan tangan. Terdapat perbezaan yang ketara antara sampel roti debunga T. laeviceps dan H. itama dari segi aktiviti antioksidan yang diukur dengan t-test ($p < 0.05$). Tiada perbezaan yang ketara dalam analisis pH bagi semua sampel madu, roti debunga dan propolis bagi T. laeviceps dan H. itama apabila diukur dengan t-test ($p > 0.05$). Dua puluh lapan komposisi kimia telah dikenalpasti dalam sampel madu T. laeviceps dan H. itama. Asid amino, gamma-butyrolactone dan steroidal saponin dikenalpasti sebagai juzuk utama kerana wujud dalam semua sampel madu.

Kata kunci: Variasi morfometrik, variasi genetik, hubungkait filogenetik, kelulut, Tetragonula spp., pendebungaan

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

<	-	less than
>	-	more than
°	-	degree
%	-	percentage
g	-	gravitational force

LIST OF APPENDICES

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Bees (Apini) and stingless bees (Meliponini) are members of the family Apidae. The stingless bees are bees with a large number of genera, about 55 genera divided into 61 subgenera, each with unique morphological, morphometric and nesting characteristics (Trianto, *et al.*, 2020; Rasmussen, 2008). The stingless bees are monophyletic groups found mainly in tropical and subtropical areas of the Americas, Australia, Africa and parts of Asia (Rattanawannee and Duangphakdee, 2020; Njoya, 2009). Stingless bees, which have no sting, live socially in perennial colonies of several hundred to several thousand individuals together in a hive (Boontop *et al.*, 2008; Michener, 2007). In Borneo, Abu Hassan (2016) and Rasmussen (2008) found 17 species from nine genera of stingless bees. These are *Geniotrigona* spp., *Heterotrigona* spp., *Homotrigona* spp., *Lepidotrigona* spp., *Lophotrigona* spp., *Sundatrigona* spp., *Tetrigonilla* spp., *Tetragonula* spp. and *Tetrigona* spp. *Tetragonula* spp. has the highest number of species compared to the other 11 genera of stingless bees in the Indo-Malay region. There are 27 species of *Tetragonula* in Indo-Malay, including nine species endemic to Borneo (Abu Hassan, 2016). In Sabah, Malaysia, *Tetragonula* spp. is generally less popular for beekeeping as it produces little honey compared to other genera of stingless bees. Therefore, this study focuses on the selected species of stingless bees of the genus *Tetragonula*, which is the smallest, and includes the study of their morphometric and morphological traits, and phylogenetic relationships, the effects of hive translocation on their genetic variability, and their ecological and economic importance.

The tropical stingless bees of the subfamily Meliponini have been studied in terms of their behaviour, ecology, genetics and evolutionary history. However, the molecular evolutionary relationships within the genus of stingless bees and correlation between morphometric traits in the rich tropical rainforests of Borneo remain unexplored. According to Gillot (2005), the important morphological characteristics of stingless bees to be studied include the colour and shape of the head, the colour and shape of the thorax, the pattern and characteristics of the wings, the length of the legs and the pattern of the abdomen. Regardless of whether they are allopatric or sympatric, the highly cryptic of *Tetragonula laeviceps, Tetragonula fuscobalteata, Tetragonula iridipennis, Tetragonula melanocephala* and *Tetragonula ruficornis* could be clearly distinguished at the genetic level based on their unique morphological traits based on their morphometric characteristics, but this has not previously investigated.

Analysis of mtDNA via nucleotide variation in the cytochrome oxidase I (COI) gene is commonly used to study the pattern of genetic variability within bee populations worldwide (Galaschi-Teixeira *et al.*, 2018; Goodall-Copestake *et al.*, 2012; Arias *et al.*, 2006). However, the study of genetic variability of wild and managed stingless bees, *T. laeviceps*, in the tropical region due to anthropogenic disturbances is still in its infancy. The growing demand for domesticated stingless bees has also resulted in the removal of many colonies from their natural forest habitats in the state of Sabah and other states in Malaysia. This study is therefore particularly important for understanding ongoing and future studies on the genetic variability of managed and wild populations of stingless bees in the tropics, which are currently lacking. In this study, the genetic structure of populations of the geographically widespread but limited dispersal of *T. laeviceps* in anthropogenic habitats was investigated using the COI gene region of mitochondrial DNA.

Stingless bees, a group of eusocial insects, play a very important role in pollinating plants, especially agricultural crops and native plants in natural habitats in tropical countries (Boontop *et al.*, 2008). Stingless bees play a crucial ecological role as pollinators of wild plants because they are polylectic (Njoya, 2009). The great diversity of their ecological and biological traits makes them ideal for comparative studies (Njoya, 2009). In Malaysia and the Asian region, 12 genera and 75 species

of stingless bees have been identified, and there are even more unknown species in Borneo (Abu Hassan, 2016). In total, there are about 27 species of *Tetragonula*, but at least 17 or more species in Borneo have not been well studied for their potential as crop pollinators. Therefore, in this study, *T. laeviceps* was selected as a model for pollination of high value crops of vanilla and pitaya flowers because its colonies are readily available from beekeepers in Sabah, Malaysia.

Meliponiculture has generally been of recent interest in Malaysia, while this activity is widespread in Mexico, Brazil, Thailand, Australia and Africa (Halcroft *et al.*, 2013; Cortopassi-Laurino *et al.*, 2006; Sawatthum, 2004). Meliponiculture enables bee farmers to generate income through the sale of stingless colonies, propolis, bee bread, honey, educational services, pollination services and agritourism activities (Halcroft *et al.*, 2013). They can produce honey in the same way as honeybees, but differ in terms of colour, taste, viscosity, and moisture content of the honey (Biluca *et al.*, 2016; de Almeida-Muradian *et al.*, 2014; Guerrini *et al.*, 2009). Recent evidence suggests that products derived from larger species of bees, such as honey, bee bread and propolis, have significant therapeutic potential (Al-Hatamleh *et al.*, 2020), but studies for smaller bee species such as *Tetragonula* spp. are limited. Until now, the potential of *Tetragonula* spp. has been neglected due to their small body size and low honey production. In this study, the quality of their products such as pollen, honey and propolis was investigated based on the phenolic and antioxidant properties.

1.2 Problem Statement

This study mainly focuses on one of the most abundant stingless bee species in Malaysia, *Tetragonula* spp. In the meliponiculture industry, bee farmers lacked information or knowledge on the potential of this species as a plant pollinator, scientific knowledge and source of high-quality bee products. Previous molecular phylogenetic studies on stingless bee species have shown that they do not conform to the traditional taxonomic groups based on morphological characters, which will be the focus of this study. Additionally, morphological identification of the stingless bee is difficult due to the phenomenon of cryptic species that show morphological

similarities particularly between species of *Tetragonula* in Borneo. The *Tetragonula* spp. have morphological characteristics that are difficult to distinguish between species, so that differences between species can be indicated as independent species or only as variations within species. Therefore, they are important for studies.

Meliponines make an important contribution to conservation by pollinating many plant species, including natural and agricultural species, and they also help to conserve native plant species. However, how the genetic variability of captive and wild stingless bees in the tropical region responds to anthropogenic disturbances is still in its infancy. Compared to other stingless bee species such as *Heterotrigona itama* and *Geniotrigona thoracica*, which are commonly selected for stingless beekeeping in Sabah, *T. laeviceps* has a limited flight radius and usually forages at a distance of about 20 to 50 m from its nest and was selected as a model organism for this study. Therefore, this species may be particularly affected by the translocation of colonies to new sites by beekeepers, which could lead to a decrease in genetic diversity and an increase in genetic differentiation between populations, as well as an overall loss of biodiversity, but further studies are needed.

Two plant species were selected to study the effectiveness of *Tetragonula* spp. as pollinators, namely vanilla (*Vanilla planifolia*) and pitaya (*Hylocereus polyrhizus*). Both vanilla and pitaya cannot self-pollinate and require pollinators such as insects to pollinate their flowers, or they are hand-pollinated. The major limitation with these two plants is that the time from flowering to blooming is very short, about a day, and unpollinated flowers usually wither, which can lead to economic losses. In pitaya, it has been observed that the flower usually opens from 6.30 pm or 7 pm, the opening of the flower is completed by 10 pm and the flower closes and begins to wilt at 2 pm the next day after pollination. As with vanilla, the plant needs special growing conditions and the formation of beans requires pollination by specialised insects (orchid bees or stingless bees), which means that pollination is done by hand in most places. The flower opens just before sunrise and reaches its maximum flowering time around midday and begins to wilt at night. If the flower is not pollinated between flowering and nightfall, it wilts and falls off the plant, usually the next morning. Vanilla needs pollination to bear fruit, but the flowers cannot self-pollinate. In other Mediterranean countries such as Mexico, they are usually

pollinated by bats, hawkmoths and orchid bees, but to date there are no known natural pollinators in the tropical region. So, there is a possibility that *T. laeviceps* can pollinate these two plants, as it is small and aggressive in foraging at short distances, which makes it interesting for studies.

Stingless bees are known among important organisms for their role in the production of natural products such as honey, propolis and the accumulation of pollen. However, the quality of honey, propolis and bee bread produced by *T. laeviceps* has been less studied compared to another preferred species for stingless beekeeping, *H. itama*. Considering the importance of natural products for the development of the meliponine industry, this study aims to investigate the potential of the antioxidant and phenolic properties of pollen, propolis and honey of this neglected species of stingless bee, *T. laeviceps*.

1.3 Justification of Study

Tetragonula spp. is the largest genus of stingless bees with many cryptic species and offers enormous potential for the meliponine industry, e.g., for scientific studies such as the study of evolutionary relationships with floral traits, pollination efficiency of high-value crops and as a source of quality natural products. *Tetragonula laeviceps* is one of about 27 species of this genus in Malaysia, many of which have yet to be described in Borneo, but whose ecological and economic importance remains largely unexplored. In addition, the growing demand for domesticated stingless bees has led to concerns that many colonies have been removed from natural forest habitats in Malaysia. The recent decline of wild and cultivated bees has also raised global concerns about the loss of important insects as pollinators, including the tropical endemic and geographically restricted distribution of bees. The use of *T. laeviceps* as a representative species for environmental indicators to demonstrate the impact of anthropogenic activities in terms of genetic variability can therefore provide important information on the status of the overall sustainable beekeeping management of stingless bees in Malaysia.

1.4 Aims

The aims of this study are as follows.

i. To determine the habitat preferences, morphometric and morphological traits, and phylogenetic relationships of selected species of the cryptic Bornean *Tetragonula*

ii. To investigate the morphological and molecular characters of cryptic speciation in *Tetragonula laeviceps*

iii. To examine the potential of *T. laeviceps* as a pollinator of vanilla and pitaya flowers

iv. To examine the antioxidant activity, phenolic compounds and chemical composition of the products of the stingless bee