AGRIKULTURA CRI Journal 1 (2) **ISSN: 2782-8816 June 2021**

STINGLESS BEES (HYMENOPTERA : APIDAE: MELIPONINI) UNDER GENUS *Tetragonula* in the PHILIPPINES

Sherwin M. Baja*, and Maria Dulce J. Mostoles

Central Bicol State University of Agriculture

San Jose, Pili, Camarines Sur, Philippines 4418

*Corresponding author: sherwin.baja@cbsua.edu.ph

Abstract — The nesting habits, morphological and genetic structures of stingless bees were assessed using the traditional taxonomic characters and BLAST analysis results in 18 populations of *Tetragonula* in the Philippines. Different structures, shapes, and colors of stingless bee entrances were recorded. Dendrogram was drawn using the measurement derived from the morphometric characters. The majority of the species build their nest in tree cavities, cemented walls, and rocks with circular-shaped entrances. Irregularity in shapes of colonies from Ogbong (Catanduanes), Calapan (Oriental, Mindoro), Alabat, and Perez (Quezon) was observed. Most colony entrances are hard and dark in color, facing east with three to 15 guard bees. Light-colored entrances were observed in colonies from Cacilles (Hernani, Samar) and Pangi (Gasan, Marinduque). Nesting habits of stingless bee species were not species-specific. The only amplicon from 16S rRNA gene reproducible in 12 colonies was analyzed. No hits found in sampled specimen from Panim-an, Caramoan, Camarines Sur, and Oriental, Mindoro (Calapan and Naujan), but, samples from Tagbilaran, Bohol, and Tagum, Davao was confirmed. In Luzon, the identified species of stingless bees were *Tetragonula iridipennis* found in Alabat and Perez (Quezon), Gasan (Marinduque), Garchitorena (Camarines Sur), Viga (Catanduanes), Placer (Masbate) and Naujan (Mindoro) while *T. laeviceps* were found in Calapan (Mindoro), and Caramoan (Camarines Sur). For Visayas, the identified species were *T. laeviceps* in Hernani (Samar), Marbuena, and Nasidman (Iloilo) while *T. sapiens* in Tagbilaran (Bohol). In Mindanao, the species was identified as *T. sapiens* in Tagum (Davao). An unidentified species was found in Paniki (Aroroy, Masbate).

Keywords **— Stingless bees, colony characterization, *Tetragonula* species gene sequencing.**

INTRODUCTION

The tribe Meliponini (stingless bees) are eusocial insects (Michener and Sakagami, 1990), which means they exhibits reproductive division of labor, cooperative brood care, and overlap of generations exhibits a pantropical distribution (Francisco et al., 2001) and are far most diverse, morphologically and behaviorally (Michener, 2013).

During explorations of the tropical world, stingless bees enjoyed a long history of discovery of many species which have been identified and described (Rasmussen, 2009). Species vary considerably in their nest architectures, which range in design for brood cells arranged in horizontal or clusters, constructed within crevices of trees, walls of human buildings, limestone cliffs, or even in the ground (Roubik, 2006; Michener, 2007, 2013; Banziger et al.,2011).

For centuries, bees made a huge impact on people worldwide, providing food (pollen and honey) and medicine (propolis) to cure a variety of ailments (Bankova, 2005). Stingless bees play an important ecological role as effective pollinators of many plant species, both wild and cultivated, and seem to be good candidates for future applications as commercial pollinators (Amano et al., 2000).

However, despite their ecological and economic benefits, limited studies have been conducted regarding the diversity, distribution, genetic classifications, and morphological variations of this group of insects. Therefore, in order for the species to complete its information and understand effectively and efficiently their habits and behaviors, it requires strongly supported studies on their nesting habits and behavioral variations by assessing its morphological and genetic response relevance (Fernandes-Salomão et al., 2002; Costa et al., 2005; and Cruz et al., 2006). The same is true in the Philippines.

Thus, the study aimed to assess the nesting habits, morphological, and genetic differences among and between the stingless bee populations occupying different island locations in the Philippines.

Information on morphological, behavioral, and genetic variation are of great importance to understand the phylogeographical patterns of the species. These informations are also relevant for the development of effective conservation strategies and to help stingless bee enthusiasts and beekeepers in selecting the best species of stingless bee for honey, pollen, and propolis production. Likewise, the results obtained in this study will help them improve techniques in colony propagation, queen rearing, drone rearing, and, possibly, artificial insemination. This research also enabled us to uncover more of the mysteries of these unique bees, and to develop their potential.

MATERIALS AND METHODS

Collection of Stingless Bee

Samples of the stingless bees were collected in 10 provinces in different islands of the Philippines. Four samples were collected from the province of Camarines Sur (Caramoan and Garchitorena , two individual feral colonies found from the islands of Masbate (Aroroy and Placer), Iloilo (Marbuena and Nasidman), Mindoro (Calapan and Naujan), Quezon (Perez and Alabat) and Gasan, Marinduque, and one individual nest from Hernani, Samar; Tagum, Davao; Tagbilaran, Bohol and Viga, Catanduanes.

The tropical climate was noted in all provinces. This type of climate was initially described as the most preferred temperature by the stingless bees

(Ramirez et al., 2010; and Solórzano-Gordillo et al., 2015). Likewise, different types of vegetation were recorded in different provinces. Majority were planted with rice (Quezon; Davao; Camarines Sur, Masbate, Mindoro, Samar, Bohol, Marinduque and Catanduanes), coconut (Quezon; Davao; Camarines Sur, Masbate, Mindoro, Marinduque and Catanduanes), banana (Davao, Samar, Bohol and Masbate), mango (Samar, Quezon, Camarines Sur, Catanduanes, and Masbate), Lanzones (Davao and Quezon) and flowering plants (Marinduque). The types of vegetation play a significant role in the formation of stingless bee colonies which are all good sources of food for the stingless bee except for rice.

Collection of Stingless Bees

Twenty-five (25) adult workers of stingless bees in every feral colony found in an area were collected, for a total of 300 samples. Stingless bees were caught by direct sampling at the entrance of their nests either by picking them one by one using forceps or by catching them using an insect net. Collected adult bees were placed in vials and preserved in 95 percent ethyl alcohol and labeled properly and set aside for morphometric study and DNA sequencing.

Voucher specimens from all sampled localities were added in the stingless bees collection of the Project on Bio-Informatics of Stingless Bee which was funded by the Department of Agriculture-Bureau of Agricultural Research (DA-BAR).

Morphometric

Morphological examination of stingless bees workers was done using the Stereoscopic Microscope (1X & 4X magnification), fitted with an ocular micrometer. Thirty-one (31) morphometric characters (19 individual characters, 12 ratios) were recorded. These parameters were adopted from the works of Sakagami (1978) and Dollin et al. (1997). Nineteen of which are from the head, malar area, gena, compound eye, ocelli, right antennae, right forewing, hind tibia, and hind basitarsus.

Morphometric Statistical Analysis

The results of morphometric studies were subjected to Stepwise Discriminant Analysis (SDA) using SPSS version 21 with p=0.05 for inclusion of a variable and p=0.10 to exclude a variable then subjected to Canonical Discriminant Analysis.

In order to show the variability of the most important quantitative characters for each species in a clear and readily comparable way, graphical tests in the form of scatter plots that illustrate the variation in parameters were applied.

Euclidean distances among populations were calculated and subjected to single linkage clustering (Nearest-Neighbor) using similar software (SPSS ver. 21) in order to construct a dendrogram that would reflect the inter-group morphometric similarities of species.

Gene Sequencing

Collected bees stored in alcohol served as biological replicates. Individual bee from each replicate was washed thrice with distilled water and dried on paper towels. The bees were placed in 1.5 mL tubes and DNA extraction was performed using the DNeasy Blood and Tissue Kit (Quiagen) following the manufacturer's protocol. The eluted genomic DNA was analyzed by agarose gel electrophoresis in a 1.3 percent agarose gel run for 30 minutes at 100V in the labnet gel X1 Ultra V-2 electrophoresis apparatus. Then, it was visualized using the alpha Innotech

Alphalmager MINI.

Extracted genomic DNA was used as a template for PCR using the Invitrogen TM PCR Super Mix. Each reaction consisted of 45 uL of the supermix, 3 uL template DNA, and 1 uL each of the forward and reverse primers.

The primers used (forward and reverse) were specific for the mitochondrial 12s (5'TAC TAT GTT ACG ACT TAT 3' and 5'AAA CTA GGA TTA GAT ACC C 3') and 16s rRNA (5'TTA CGA TGT TAT CC TAA 3' and 5' CGC CTG TTT ATC AAA AAC AT 3'), cytochrome oxidase I (CO1) (5' TGA TCA AAT TTA TAA T 3' and 5' GGT AAA ATT AAA ATA TAA ACT TC 3'), cytochrome oxidase II (COII) (5' ATT AGA TGT TGA TAA TCG 3' and 5'ACA AAT TTC TGA ACA TTG 3'), or the NADH dehydrogenase (NADDH) (5' CTA AAG TTG ATG AAT GAA CTA AAG 3' and 5' GCT CAT GTT GAA GCT CC 3') gene fragments.

PCR was done using the Veriti 96 well Thermal Cycler (Applied Biosystems). PCR products were checked for amplification by subjecting them to agarose gel electrophoresis in a 2% agarose gel run at 100 V for 30 minutes. Successfully applied gene fragments were sent to 1st BASE DNA Sequencing Services for single-pass capillary sequencing.

Basic Local Alignment Search Tool (BLAST) analysis was used to determine the similarities and relationships between and among species.

Identification and Confirmation of Results

Confirmation of the species of the stingless bee samples collected was facilitated by the results of the morphometric study and the genetic sequences with the help of different books, catalogs, and taxonomic keys provided by many authors. If confusion arises, the nest characters were considered to identify the species. After the bees have been identified, these were plotted into a map using the Geographical Information System. The map shows the locations and the species of the stingless bees collected in the different islands of the Philippines.

RESULTS AND DISCUSSION

Species Identified using Nesting Sites and Hive Entrances

Nesting Sites of Stingless Bees. A total of 18 colonies of stingless bees were collected and used in the study. The majority of these colonies were collected in Luzon (13 colonies), Visayas (4 colonies), and Mindanao (1 colony). All colonies were found in hard, solid, and permanent objects like in crevices of the living tree trunk, decaying logs, cemented wall, and rocks, whereas others were found in some parts of human houses, such as wall crevices, drawers, and bamboo cliffs. In particular, each stingless bee showed specific nest requirements according to its habitat quality.

It was found that in Luzon, the highest number of colonies were found living in cavities of tree trunks and walls of buildings, followed by colonies living in crevices of rocks and decaying logs. In Visayas, nesting sites of colonies were in tree trunks, dead logs, buildings, and crevices while the colony from Mindanao nested inside an old and damaged table. The study revealed that nesting habits of stingless bees found in Camarines Sur (Caramoan and Garchitorena), and Masbate (Placer) were recorded living in a cavity of *Avecennia alba* (myiapi). Likewise, a colony from Tagbilaran, Bohol was found similar in nesting habits in colonies found in Masbate, Marinduque, Oriental Mindoro, and Quezon. All these colonies were recorded living in crevices of building walls. Likewise, three colonies were

found to live in stone crevices in Mindoro, Marinduque, and Camarines Sur.

Nest Entrances of Stingless Bee Colonies. Different shapes, colors, and rigidity of nest entrances of stingless bees were observed. Most commonly, tubular, ovular, round-ringed, and irregular entrances were found in different islands in Luzon. Similar shapes of entrances were recorded in Camarines Sur (Caramoan and Garchitorena), Iloilo (Marbuena and Nasidman), Bohol, and Marinduque, with round and tubular types. However, in the islands of Quezon and Catanduanes, irregular entrances were recorded.

The color of their entrances varied from light brown to black (Figure 1-3). Light-colored entrances, however, were observed in Camarines Sur, Iloilo, Samar, and Marinduque, while dark colored entrances were observed in colonies collected in Davao, Bohol, Quezon, and Camarines Sur. All entrance holes, on the other hand, were observed facing east with 3 to 15 bee guards.

It was recorded that all colonies collected in Visayas and Mindanao islands were characterized by soft rigidity. A similar feature was observed in six colonies collected in Luzon (Camarines Sur, Mindoro, Catanduanes, Marinduque, Masbate, and Quezon).

Based on the nesting sites of colonies, the identified species belong to the *Tetragonula* group. Since most of the species were cavity nesters, these species belongs to *laeviceps* or *iridipennis* group. This study corroborates with the results of Gajanan et al. (2005) which recorded nesting sites of *T. iridipennis* in crevices of tree trunks, stone and mud walls, corners of walls, and termite mounds. This further corroborates with recent studies in the Bicol region by Davila , 2017; Penaverde , 2017; and Esplana ,2017 that the cavity nesters found in different areas in the region were identified as *T. iridipennis, T. laeviceps, T. sapiens, and T. fuscobalteata.*

Species Identified using Morphometrics

Morphological characteristics. The workers of each bee species have varied morphological characteristics. Out of 31 morphological characters analyzed, 27 of which were identified by the stepwise analysis as significant variables that had considerable contributions to the intra- and interspecific separation of the group samples. Seventeen for individual characters such as HW, LOD, SC, BL, GW, F4W, HTL, ML, WL2, MOD, IOD, OOD, F4L, HTW, EW, EL, and HBW) and 10 for ratios (SC/EL, ML/FW, HTW/HTL, IOD/OOD, FL/FW, WL2/HW, GW/EW, LOD/MOD, HBW/HTW, and EL/MOD) were noted.

In the study, the smallest worker stingless bees were recorded in Aroroy, Masbate (unidentified species of *Tetragonula*) (3.496 + 0.17 mm), while the biggest was found in Pangi, Gasan, Marinduque (*T. iridipennis*) (4.62 + 0.32 mm). However, these variations in body length were significant between and among stingless bee populations. The worker body size has been generally considered as an adaptation to foraging activity and floral resource exploitation. Larger species, on the other hand, have a greater capacity to migrate between and among environmental fragments, while the smaller stingless bees depend only on the available resources within their jurisdiction.

General Characteristics. The workers of each bee species found in the study showed varied morphological characteristics. *T. laeviceps* have a 3.89 - 4.30 mm long body, of which the smallest bees were collected in

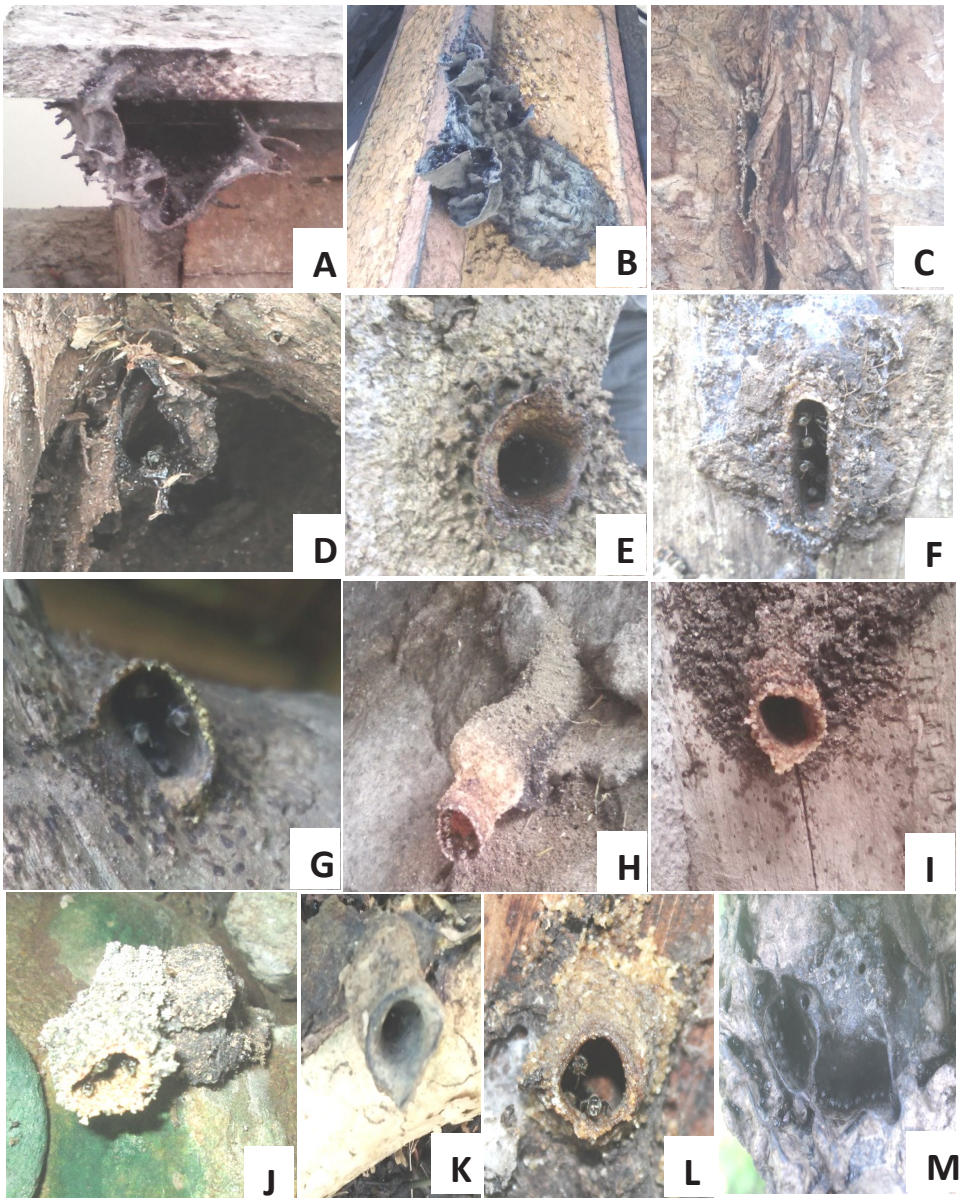

Fig. 1. Nest entrances of *Tetragonula* in Luzon Islands: A. Alabat, Quezon; B. Perez, Quezon; C. Viga, Catanduanes; D & E. Garchitorena, Cam. Sur; F& G. Aroroy & P lacer, Masbate; H & I. Gasan, Marinduque; J & K.Caramoan, Cam.Sur and L&M. Calapan and Naujan,

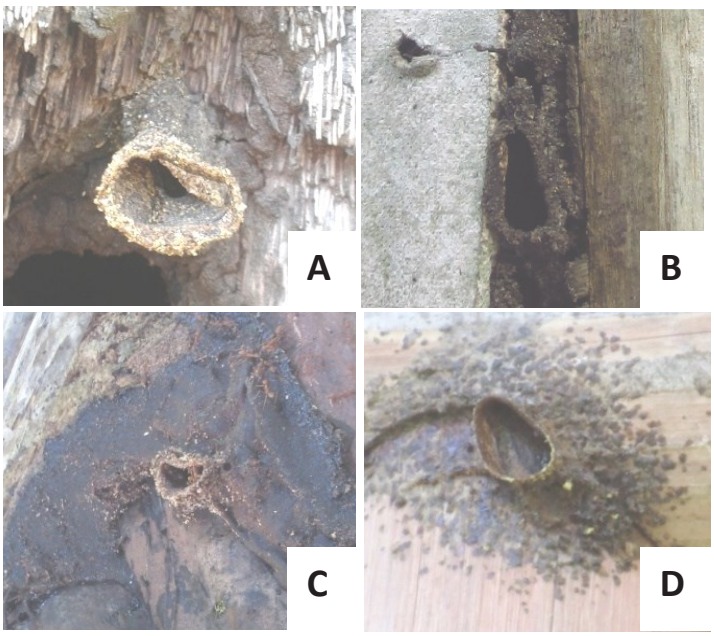

Fig. 2. Nest entrances of *Tetragonula* found in Visayas Islands (A. Hernani , Samar, B. Tagbilaran, Bohol; C. Marbuena Island, Ajuy, Iloilo; and D. Nasidman, Iloilo).

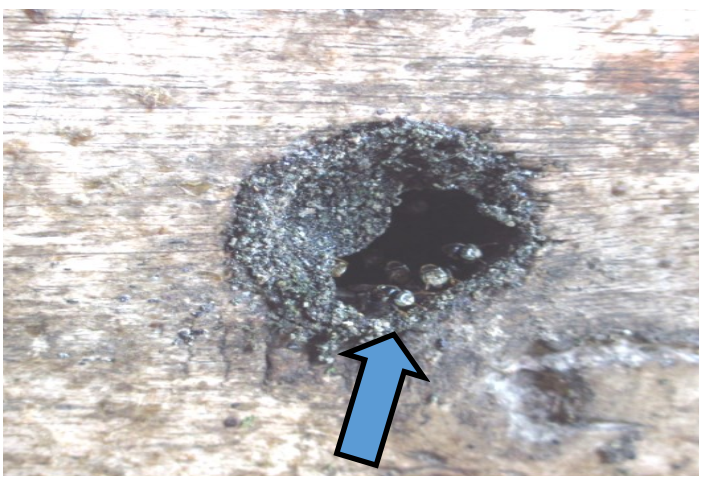

Fig. 3. Nest entrances of *Tetragonula* found in Tagum, Davao (Mindanao).

Fig. 4. Adult workers of *Tetragonula* species.
A. *T. laeviceps*; B. *T. sapiens*; C. *T. iridipennis*;
D. *Tetragonula* sp. (Unidentified species).

Caramoan, Camarines Sur while the biggest was found in Marbuena Island, Ajuy, Iloilo. Their body is characterized by black color, mesoscutum is hairy, the vertex is blackish and not banded, and anterior hind tibia is hairy (Figure 4A).

Workers of *T. sapiens* have 3.8 - 6.0 mm long body, black colored body, brown metasoma, first and the second tergum blackish-brown and blackish in the apical black mesoscutum with dark to blackish-brown hairs (Figure 4B).

Workers of *T. iridipennis* have 3.50 - 4.60 mm long body with black clored body. Mesoscutum is hairy, vertex blackish with 4-6 banded and anterior hind tibia with dark to blackish-brown hairs, while the posterior is brownish-yellow (Figure 4C).

Workers of unidentified species of *Tetragonula* have 3.3— 3.80 mm long body, the smallest among the species collected in different islands of the Philippines. Characterized by an enlarged compound eye, black-brown body color, brown metasoma, first and the second tergum blackish-brown, and blackish in the apical, slightly banded. The anterior hind tibia is characterized by blackish-brown hairs, while the posterior is blackish-yellow (Figure 4D).

Head and its Appendages.
From the results of the stepwise analysis, the majority of the variables that best discriminate the group samples were found in the head (11 characters). These are the maximum width of head, lower inter-orbital distance, scape length, the maximum width of gena, length and width of flagellomeres IV, width and length of compound eyes, length of the malar area, maximum interorbital distance, interocellar andocellocular distance. It was seen

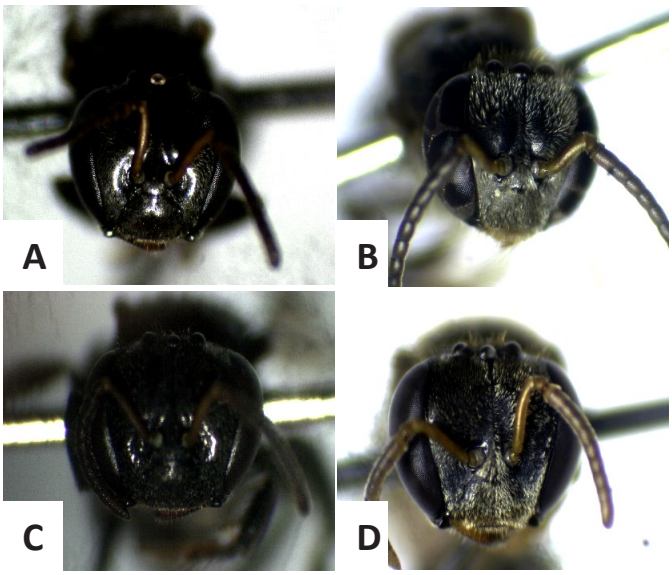

Fig. 5. Head structure of *Tetragonula* species. A. *T. laeviceps;* B. *T. sapiens;* C. *T. iridipennis;* and D. *Tetragonula sp.* (Unidentified species).

that workers of *T. iridipennis* as well as and *T. laeviceps* have overlapping characters. The width of their head, length of the malar area, lower inter-orbital distance, interocellar and ocellocular distance, and the length and width of flagellomeres IV are identical with 1.74, 0.25, 1.18, 0.26, 0.53, 0.13, and 0.13 mm, respectively (Figure 5).

However, the maximum width of gena (0.25 mm) and interocellar distance (0.26 mm) are the only characters that showed similarity between *T.laeviceps* and *T. sapiens*. This means that the above mentioned characters were not useful in identifying the species of *T. iridipennis, T. laeviceps* and *T. sapiens.* On the other hand, it was noted that the length and width of their compound eye, the maximum interorbital distance, and the length of scape became an independent variables among and between the three species collected (Figure 6).

This indicates that *T. iridipennis, T. laeviceps* and *T. sapiens* could be easily separated using these morphological characters.

Thorax and its Appendages. Among the 27 characters identified by the stepwise analysis assignificant variables in discriminating the stingless bee populations in the Philippines, four have been recorded in the thorax .

These are the length and width of hind tibia, maximum width of hind basitarsus and the distance between *M-Cu* bifurcation and basal tip of marginal cell of forewing (Figure 7).

However, it was recorded that only the width of hind tibia and basitarsus were identified as distinguishable characters among the three species, *T. iridipennis, T. laeviceps* and *T. sapiens,* since the length of their hind tibia were recorded similar in *T. laeviceps* and

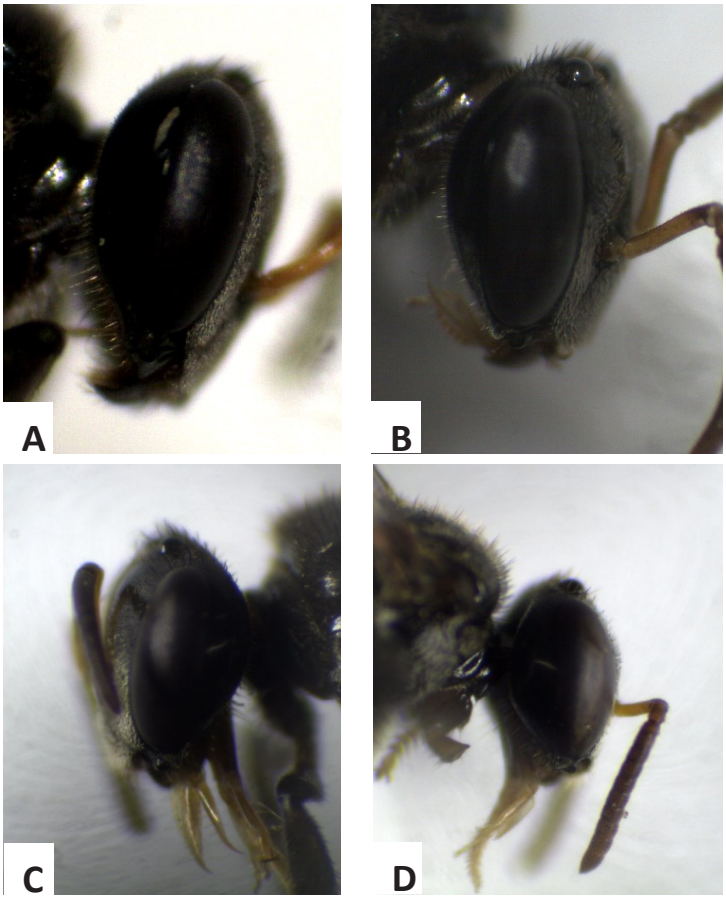

Fig. 6. Compound eye of *Tetragonula* species A. *T. laeviceps; B. T. sapiens; B. T. iridipennis;* and *D. Tetragonula* sp. (Unidentified species).

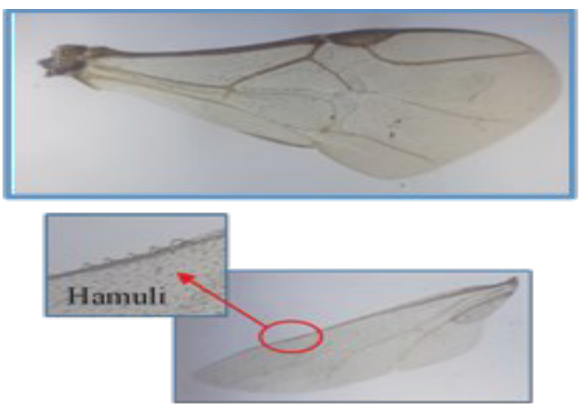

Fig. 7. Forewings (A) and Hindwings (B) of stingless bee populations in the Philippines.

T. sapiens (1.64 mm). Likewise, the distance between M-Cu bifurcation and basal tip of marginal cell of forewing were recorded identical in *T. iridipennis* and *T. sapiens* (1.14 mm).

Similarly, it was recorded that all examined specimens showed a clear, monotone and transparent types of membranous wings (Figure 7A). Likewise, it was found out that all collected bees had five (5) *hamuli* (Figure 7B). These characteristics were first recognized as distinct features for *Tetragonula* species.

Canonical Discriminant Analysis

There are four distinct clusters revealed for the entire population of the stingless bees from the scatter plot for the 1st and 2nd canonical discriminant functions analysis (CDA), based on the reduced number of characters resulted from the stepwise analysis (Figure 8). The two functions, however, were correctly classified 83.3 percent of the original groupings.

The first cluster which shares their common characters were recorded in groups 13, 14, and 18, which were collected in Ogbong, Viga, Catanduanes and Pangi, Gasan, Marinduque (1&2). These group of bees, however, share their common morphological characters in terms of the size of their head (width) (1.9 mm), compound eye (width) (0.5 mm), length of scape (seen laterally) (0.5 mm), lower interorbital distance (1.2 mm) and the distance between M-Cu bifurcation and the basal tip of the marginal cell (1.2 mm).

Unlike the latter cluster, groups 8, 9, 10, 11, and 12 were more closely related to each other. This cluster showed the overlapping of the group of *T. laeviceps* and *T. sapiens* which were found from the islands of Iloilo (Marbuena and Nasidman), Samar (Hernani), Davao (Tagum), and Bohol (Tagbilaran). These groups, however, share their common characteristics in terms of the size of their compound eye (EL) (1.2 mm), interocellar distance (IOD) (0.3 mm), ocellocular distance (OOD) (0.5 mm), length of malar area (ML) (0.3 mm) and the distance between *M-Cu* bifurcation and the basal tip of the marginal cell of the forewing (WL2) (1.1mm). Likewise, similar measurements were recorded in relation to the following: (1) wing length to head width (WL2/HW) (0.6 mm), (2) lower interorbital distance to maximum interorbital distance (LOD/MOD) (1.1 mm), (3) interocellar distance to ocellocular distance (IOD/OOD) (0.5 mm), (4) malar length to flagellomere (IV) width (ML/FW) (2 mm).

However, eight samples 1, 2, 3, 4, 6, 7, 15, 16, and 17) occupied the third cluster, which slightly overlapped with each other and nearest to the centroid. Colonies from groups 1 to 7 were found in the islands of Camarines Sur (Caramoan and Garchitorena) and Mindoro (Calapan and Naujan) of which two species *T. iridipennis* and *T. laeviceps* were identified while the remaining groups were found in the islands of Masbate (Placer) and Quezon (Alabat and Perez) which are recorded belonging to *T. iridipennis* populations.

Furthermore, group 5 was distinctly separated from the other groups. Sample specimens from group were collected from the island of Masbate (Aroroy). This group was recorded as the farthest among all distances between any groups. These bees, however,

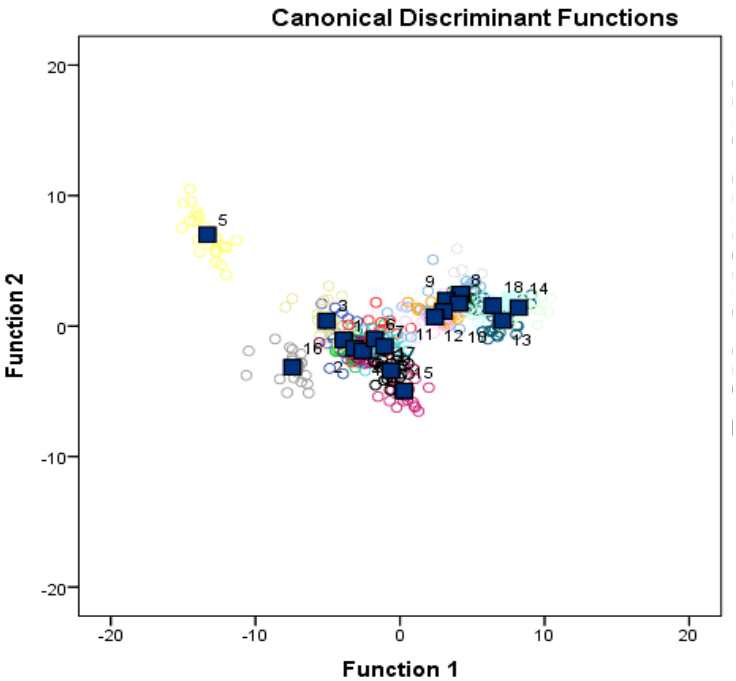

Fig. 8. Scatterplot of the Canonical Discriminant Analysis (CDA) showing the clustering of *Tetragonula* sp.

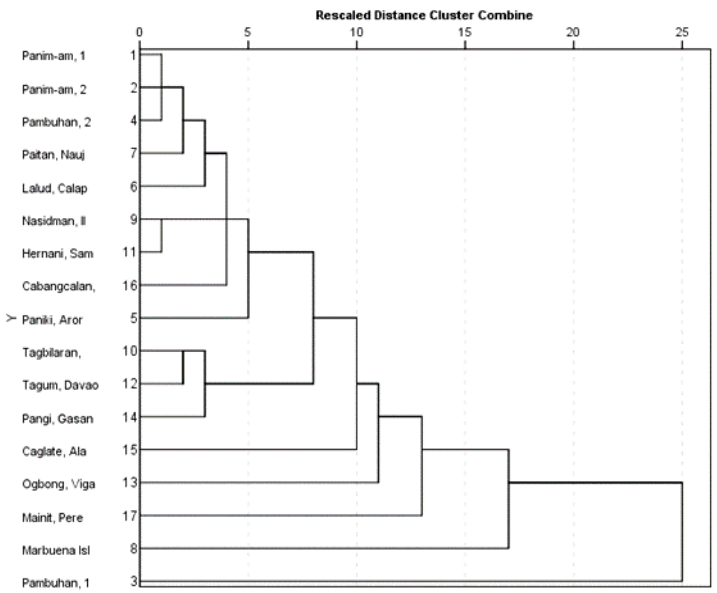

Fig. 9. Neighbor-joining dendogram generated from the Euclidean distances between the centroids of CDA indicating probable distribution of *Tetragonula* populations in the Philippines.

were morphologically identified as belonging to the genus *Tetragonula.* No similar record, however, matches their unique characteristics which means that a possible new species of *Tetragonula* was found on the island of Masbate.

The graphical representation of CDA scores shows a number of subpopulations within the species in the different collection sites. Ecotype distributions seem to be pulling along latitudinal gradient under the influence of a number of environmental variables.

Species Group Relationships and Similarities

The results of the neighbor-joining dendrogram generated from the Euclidean distances between the centroids of CDA highlighted the species relationship and similarities of various sample populations of stingless bees collected in the different islands of the Philippines. The graphical representation show clear proximities of various populations with each other (Figure 9). Among the group populations, sample specimens from Camarines Sur (Caramoan 1 & 2 and Garchitorena 1) and Mindoro (Naujan and Calapan) were recorded as the closest group. Thus, specimens from this group were identified to belong to *T. iridipennis* populations.

The dendrogram separated the adult workers of stingless bees from Tagbilaran, Bohol & Tagum, Davao. This group was confirmed as belonging to *T. sapiens.* Similar observations were noted in samples from Nasidman, Iloilo, and Hernani, Samar which are identified as belonging to *T. laeviceps.* Distances from these groups were, however, found in a range of 0 to 5.

The results indicated that the morphological characters are useful in the separation of the two species *T. laeviceps* and *T. sapiens.* But for *T. iridipennis* group, the results of the dendrogram showed that their populations were not separated due to several morphological variations. Therefore, the separation of species should use the nest structure, and DNA sequencing besides morphological characters.

Species Identified using Gene Sequencing

Out of 18 stingless bees samples, only 12 samples were submitted for DNA sequencing analysis due to insufficient numbers. Samples from groups 13 to 18 were collected in Ogbong, Viga, Catanduanes, Pangi, Gasan, Marinduque, Caglate, Alabat, Quezon, and Mainit, Perez, Quezon were not included in DNA sequence analysis.

Basic Local Alignment Search Tool (BLAST) analysis revealed that only amplicon from 16S RNA genes was reproducible in 12 colonies analyzed. However, no hits were found in samples from Panim-an, Caramoan, Camarines Sur and Oriental, Mindoro (Calapan and Naujan). This is an indication of a new possible strand or subspecies as the output of the study.

The BLAST searches showed that forward sequence from Tagbilaran, Bohol has 97 percent identify with the 16S rRNA genes found in the Old World Stingless bee, *Tetragonula sapiens.* Likewise, an almost similar percentage was recorded in samples from Tagum, Davao with 97.3 percent (Forward) and 92.1 percent (Reverse) identity with 16S rRNA genes found in the same species. This means that samples from these islands are confirmed to belong to *T. sapiens.*

However, the two-sample specimens from Panbuhan, Garchitorena, Camarines Sur (1&2), showed both forward (92.3%) and reverse (90.2 & 92.6%) sequence similarity with the 16S rRNA genes found in *Tetragonula pagdeni* while the samples from Paniki, Aroroy, Masbate were found 94.5 and 94.1 percent (Forward and Reverse) in the old world stingless bees, *Tetragonula clypearis*. However, the rest of the samples collected in the different islands of the Philippines shared their genetic similarity in *T. sapiens*.

No studies on the gene sequencing of stingless bees currently exists. Moreover, it is important that the information generated must be submitted to the database for the BLAST Analysis of stingless bees.

Species Confirmation

Although the nest of stingless bees is often used as a key feature to solve taxonomic problems (Dollin et al*.,* 1997), the results of the study suggest that nesting habits of stingless bees and the structures of their entrances were not species-specific due to close similarities and habits.

Likewise, due to the absence of reliable structural characters in the workers (Sakagami, 1978), sorting of stingless bees species collected became the main problem in the study. However, Sakagami (1978) suggested that the classification must depend on the size, coloration, proportion, and pilosity, which the study followed and used together with the results of BLAST analysis.

Thus, the results of the study revealed that there were three (3) species and an unidentified species of stingless bees where the samples were classified or identified. All species noted belong to the genus *Tetragonula*. The genus was characterized by the small body size and could be found throughout the world with 500 species (Ramirez et al.,2010). The genus *Tetragonula* were likewise recorded in the continental Asia, Sri Lanka, India, Southeast Asia , Thailand, Malaysia (Sakagami, 1978; Rasmussen and Michener 2010).

Three species of stingless bees were found in the provinces of Camarines Sur and Mindoro (*T. laeviceps* and *T. iridipennis)* while the rest of the species were distributed throughout the country.

In the study, the highest number of colonies found was *T. iridipennis* (9 colonies), followed by *T. laeviceps* and *T. sapiens* with six and two colonies, respectively.

In particular, each species showed specific nest requirements

based on their habitat quality. The *T. sapiens* colonies were exclusively found in the parts of the houses, such as wall and drawer while colonies of *T. laeviceps* and *T. irridipennis* build their nest in hard, solid, unbreakable, and permanent objects like in the crevices of the living tree trunk, decaying logs, cemented wall, and rocks.

The highest number of *T. iridipennis* colonies were found in crevices of tree trunks (Miyapi and Narra) (3 colonies) followed by decaying logs (2 colonies), stone cavity, wooden wall, cemented post, and fern. However, no specific nesting sites were recorded in *T. laeviceps*.

The characteristics of nest entrances of *T. laeviceps* are tubular and round-ringed. The colors are light brown, brown, and black with both soft and hard rigidity. Nest entrances of *T. iridipennis* are characterized by irregular shapes. Others are tubular, round-ringed, light to dark brown in color, and soft and hard rigidity. Circular shape, black in color, and soft rigidity are characters of nest entrance of *T. sapiens,* while, dark brown in color, ovular in shape, and soft rigidity are characters observed in the nest entrance of an unidentified species. However, all entrance holes were observed facing east with 3 to 15 guards. However, considering the BLAST results, it could be inferred that only samples from Tagbilaran (Bohol) and Tagum (Davao) were confirmed similar to *T. sapiens*. However, samples from Panbuhan (Garchitorena), Paniki (Aroroy, Masbate), Marbuena and Nasidman (Iloilo) and Hernani (Samar) were not similar to *T. pagdeni,T. clypearis)* and *T. sapiens,* respectively, since the values are less than 97 percent.

The results indicated that sample specimens from these groups are confirmed to be *Tetragonula* Moure considering the available data base for the stingless bees.

On the other hand, the results of the morphometric analysis revealed that five specimens collected in Caramoan, Camarines Sur, Paitan, Naujan, Oriental Mindoro, Hernani, Samar, Marbuena, and Nasidmen Iloilo, had great similarity on workers of *T. laeviceps*. Likewise, nine populations were similar to *T.iridipennis* and two groups similar to *T. sapiens* and one unidentified species coded as *Tetragonula* sp.

Using the QGIS Version 2.16.1 program, different locations where stingless bee samples were collected have been plotted and a distribution map was generated. The distribution map showed the areas where specific species of stingless bees could be found (Figure 10).

Presence of *T. sapiens* was noted in Tagbilaran, Bohol, and Tagum, Davao which were collected in the crevices of the wall and file cabinet inside the house. However, *T. iridipennis* found in the island of Masbate (Placer), Camarines Sur (Garchitorena), Oriental Mindoro (Calapan), Catanduanes (Viga), Marinduque (Gasan), and Quezon (Alabat and Perez), which are collected in cavities of Miyapi tree, decaying wood, narra, cemented wall and rocks.

T. laeviceps, however, was recorded in Caramoan (Camarines Sur) Paitan, Naujan (Oriental Mindoro) Hernani (Samar,) Marbuena, and Nasidman (Iloilo),

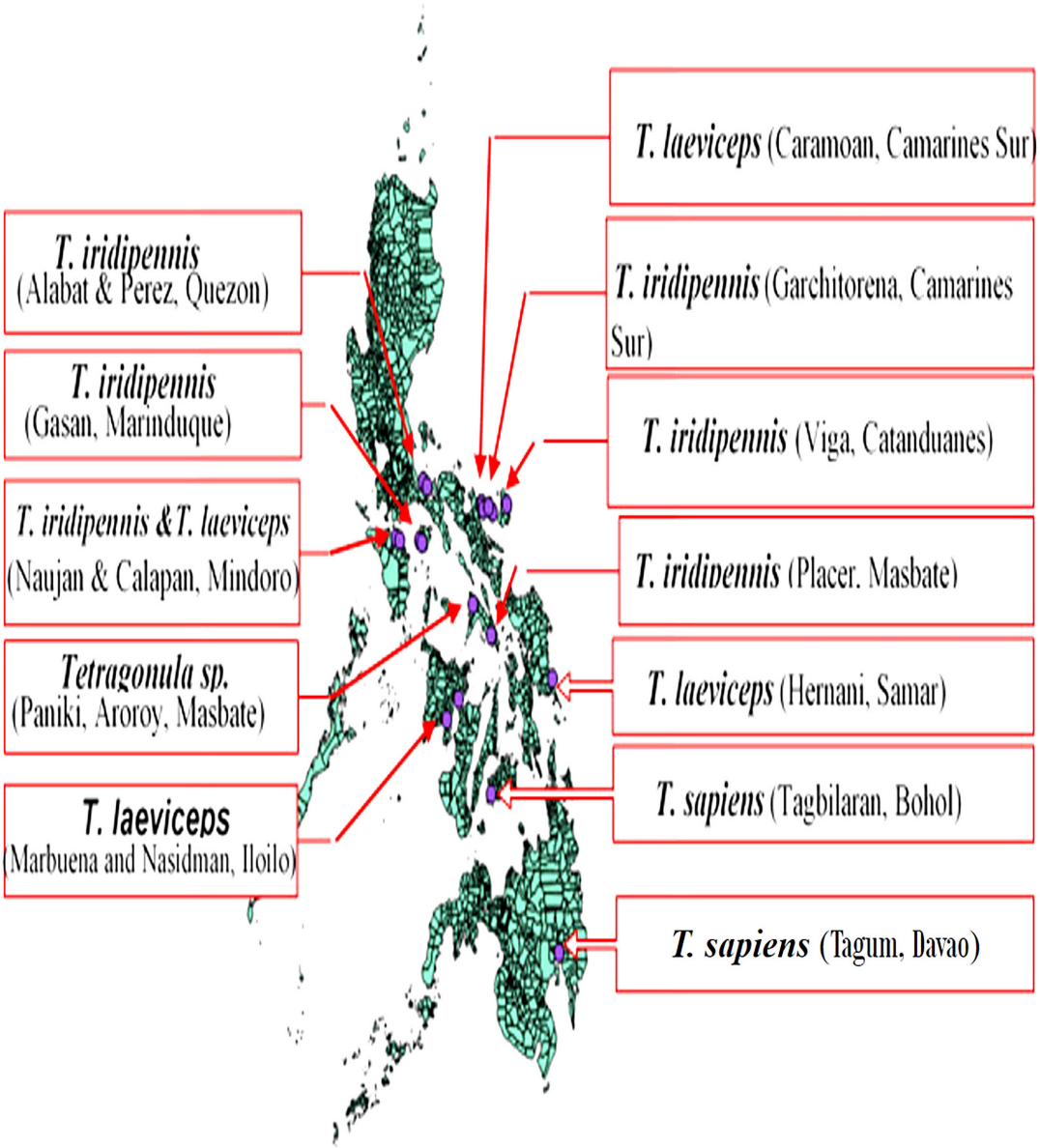

Fig. 10. Philippine map showing the collection sites with the identified stingless bees species.

while in Aroroy (Masbate) , an unidentified species was observed.

The distribution map would be useful in understanding the existence of stingless bees in a certain area and the possible effects of geographical differences on their morphological features.

CONCLUSIONS

The morphometric characters were used as an effective tool for discriminating *Tetragonula* species.

Samples from Masbate (Aroroy & Cabangcalan) were distinctly separated from the other groups.

The only amplicon from 16S rRNA genes was reproducible in 12 colonies analyzed. No hits were found in samples specimen from Paniman, Caramoan, Camarines Sur (1&2), and Oriental, Mindoro (Calapan and Naujan), however, samples from Tagbilaran, Bohol, and Tagum, Davao were confirmed.

In Luzon, the identified species of stingless bees in the islands was *T. iridipennis* found in Alabat and Perez (Quezon), Gasan (Marinduque), Garchitorena (Camarines Sur), Viga (Catanduanes), Placer (Masbate), and Naujan (Mindoro) while *T. laeviceps* were found in Calapan (Mindoro), Caramoan (Camarines Sur). For Visayas, the identified species were *T. laeviceps* in Hernani (Samar), Marbuena, and Nasidman (Iloilo) while *T. sapiens* in Tagbilaran (Bohol). In Mindanao, the species was identified as *T. sapiens* in Tagum (Davao). An unidentified species was found in Paniki (Aroroy, Masbate).

Stingless bees preferred cavities to build their colonies in more secured, hard, solid, unbreakable, and permanent objects, nesting habits of the stingless bees collected were not species-specific.

To solve problems regarding stingless bees similarities and relationships, the following recommendations are: 1) There is a need to study on adaptations and distribution of the stingless bees considering its morphology, nesting habits, and nest architecture; 2) The results of the molecular analysis of the different stingless bees species found in the study must be inputted in the database for Indo-Malayan bees, however, further sequencing of the whole genome using mitochondrial analysis must be done to complete the data on the stingless bees particularly in the Philippines; 3) It is also recommended that another similar study be conducted using similar methodology focusing the whole Islands of the country.

ACKNOWLEDGMENT

The authors extends their gratefulness for the funding support provided by the Department of Agriculture Bureau of Agricultural Research. They are grateful to the support provided by the beekeepers and people of the different island provinces visited during the collection of feral colonies.

REFERENCES

Amano K, Nemoto T. and Heard T 2000. What are stingless bees, and why and how to use them as crop pollinators? A review. Jpn. Agric. Res. Q. 34: 183-190.

Bankova, Vassya. 2005. Recent Trends and Important Development in Propolis Research. 2(1) : 2152.

Banziger H, Pumikong S, Srimuang K-O. 2011. The remarkable nest entrance of tear drinking *Pariotrigona klossi* and other stingless bees nesting in limestone cavities (Hymenoptera: Apidae). J. Kansas Entom. Soc. 84: 22–35.

Camargo J.M.F., Pedro S.R.M. 1992. Sytematics, phylogeny and biogeography of the Meliponinae (Hymenoptera, Apidae): a mini-review, Apidologie 23, 509–522.

Costa R.G., Tavares M.G., Dias L.A., Campos L.A. 2005. Isoenzyme variation in *Melipona rufiventris* (Hymenoptera: Apidae, Meliponina) in Minas Gerais State, Brazil, Biochem. Genet. 43, 49–58.

Cruz D. O., Jorge D.M., Pereira J.P., Torres D.C., Soares C.A., Freitas B.M., and Grangeiro T.B., 2006. Intraspecific variation in the first internal transcribed spacer (ITS1) of the nuclear ribosomal DNA in *Melipona subnitida* (Hymenoptera, Apidae), an endemic stingless bee from northeastern Brazil, Apidologie 37 (2006) 376–386.

Danaraddi, C. S., and Viraktamath S, 2009. Morphometrical studies on the stingless bee, *Trigona iridipennis* Smith. Karnataka J. Agric. Sci. 22 (4): (796-797.

Davila, Ludovic Dale M. 2017. Stingless Bees (Hymenoptera: Apidae; Meliponini) of Burias Island, Masbate. Unpublished Undergraduate Thesis. Central Bicol University of Agriculture. 50 pp.

Devanesan, S., Shailaja, K. K., Raakhee, M., Bennet, R. and Premaila, K. S. 2003. Morphometric characters of the queen and worker of stingless bees, *Trigona iridipennis* Smith. Insect Environment 9 (4):154-155.

Dollin, Anne E., Dollin, Leslie J. & Sakagami, S.F. 1997. Australian stingless bees of the genus *Trigona* (Hymenoptera: Apidae). Invertebrate Taxonomy 11 (6): 861-896.

Eltz, T., C. A. Bruhl, Z. Imiyabir And K. E. Linsenmair. 2003. Nesting and nest trees of stingless bees (Apidae: Meliponini) in lowland dipterocarp forests in Sabah, Malaysia, with implications for forest management. Forest Ecology and Management, 172: 301–313.

Esplana, Jasmin C. 2017**.** Stingless Bees (Hymenoptera: Apidae Meliponini) At Ocampo, Camarines Sur. Central Bicol State University of Agriculture. 48 pp.

Fernandes-Salomão T.M., Muro-Abad J.I., Campos L.A.O., and E.F. Araújo.2002. Mitochondrial and nuclear DNA characterization in the *Melipona* species (Hymenoptera, Meliponini) by RFLP analysis. Hereditas 137: 229–233.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Francisco FDO, Silvestre D, Aria Mc 2001. Mitochondrial DNA characterization of five species of *Plebeia* (Apidae: Meliponini): RFLP and restriction maps. Apidologie 32 (2001) 323–332.

Foster, J.B. 1964. Evolution of mammals on islands. Nature, 202, 234–235.

Gajanan, S. 2005. The Nest Architecture of Stingless bee, *Trigona* iridepennis. Indian Bee Journal 67 (1 & 2): 36-40.

Heard, TA .1999. The role of stingless bees in crop pollination. Annu. Rev. Entomol. 44: 183-206.

Heaney, L.R. 1978. Island area and body size of insular mammals: evidence from the tri-colored squirrel (Calliosciurus prevosti) of Southwest Africa. Evolution 32: 29–44.

Jain, S. K. 1975. Population structure and the effects of breeding system. - In: Frankel, O. H. and Hawkes, J. G. (eds). Crop genetic resources for today and tomorrow. Cambridge Univ. Press, London. 15-36.

Jongjitvimol T. and Wattanachaiyingcharoen W, 2007. Distribution, Nesting Sites and Nest Structures of the Stingless Bee Species, *Trigona collina* Smith, 1857 (Apidae, Meliponinae) in Thailand. The Natural History Journal of Chulalongkorn University 7(1): 25-34.

Kuberappa, G. C., Gajanana, S. Mohite and R. N. Kencharaddi 2005. Bio-metrical variations among populations of stingless bees, in Karnataka. Indian Bee J. 67(3&4): 145-149.

Kumar S., Stecher G., and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics version 7.0 for bigger data sets. Molecular Biology and Evolution 33:1870-1874.

Librado, P., Rozas, J. 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Loveless, M. D. and Hamrick, J. L. 1984. Ecological determinants of genetic structure in plants populations. Ann. Rev. Ecol. Syst. 15: 65-95.

Lundqvist E. 2002. Genetics and ecology of natural populations. (Doctoral dissertation). Department of Molecular Biology / Division of Genetics SE-901 87 Umea, Sweden

Lomolino, M.V. 2005. Body size evolution in insular vertebrates: generality of the island rule. J. Biogeogr 32: 1683–1699 .

Michener, CD 1974. The Social Behavior of the Bees, a Comparative Study. Belknap Press of Harvard University Press, Cambridge.

Michener , CD. 2000. The bees of the world, The John Hopkins University Press, Baltimore and London.

Michener , CD and SF Sakagami. 1990. Classification of the Apidae (Hymenoptera). Appendix: *Trigona genalis* Friese, a hitherto unplaced New Guinea species. Univ. Kansas Sci. Bull. 54: 75-164.

Michener, CD. 2007. The Bees of the World [2nd Edition]. Johns Hopkins University Press; Baltimore, USA. xvi 953 pp.

Michener , CD. 2013. The Meliponini. 3–17 pp. In Vit P, Pedro SRM, Roubik DW, eds. Pot Honey: A Legacy of Stingless Bees. Springer Verlag; Berlin, Germany. xxviii 654 pp.

Mostoles, MDJ, Buenaagua RR, Pasiona, LC, Del Rosario, AB. Bien, R, Servilla, EN, Espejo, MM. 2015**.** Performance of stingless bees (*Tetragonula biroi* Freise) in Different Hives Under Different Ecosystems. The Philipp. Entom. 29(2): 123-135.

Mostoles, MDJ and S.M. Baja. 2017. Colony characterization and Amplicon Sequencing of Stingless Bees (*Tetragonula biroi*).Proceedings of the Conference, Malaysia. p. 27.

Peñaverde, J. B. 2017**.** Stingless Bees (Hymenoptera: Apidae; Meliponini) of Pili, Camarines Sur. Unpublished Undergraduate Thesis Central Bicol State University of Agriculture. 51 pp.

Rasmussen C. and S. Cameron. 2007. A molecular phylogeny of the Old World stingless bees (Hymenoptera: Apidae: Meliponini) and the non-monophyly of the large genus *Trigona*. Systematic Entomology 32: 26–39.

Rasmussen C. 2009. Molecular Phylogeny of Stingless Bees: Insight into divergence times, Biography and Nest Architecture Evolution (Hymenoptera: Apidae: Meliponini). Dissertation, M.S., Aarhus University, Urbana, Illinois. 206-232.

Ramirez, S.R., Nieh J.C., Quental, T.B., Roubik, D.W., Imperatriz-Fonseca V.L., and Pierce N.E. 2010. A molecular phylogeny of the stingless bee genus Melipona(Hymenoptera: Apidae). 519–525.

Roubik, D.W., 2006. Stingless bee nesting biology. Apidologie 37, 124–143.

Sakagami, S. F. 1978. Tetragonula Stingless bees of the Continental Asian and Srilanka (Hymenoptera: Apidae). J. Fac. Sci. Hokkaido Univ. Ser. Zool. 21: 165-247.

Slaa Ej, Sanchez La, Sandi, M and Salazar, W 2000. A scientific note on the use of stingless bees for commercial pollination in enclosures. Apidologie 31: 141-142.

Slaa Ej, Chaves Las, Malagodi-Braga Ks and Hofstede, Fe 2006. Stingless bees in applied pollination: practice and perspectives. Apidologie 37: 293-315.

Slatkin, M. 1985. Gene flow in natural populations. Ann. Rev. Ecol. Syst. 16: 393-430.

Solórzano-Gordillo, E. De J., Cabrera-Marín, N. V., Mérida, J., Vandame, R. & Sánchez, D. 2015. Genetic diversity of two stingless bees, *Trigona nigerrima* (Cresson 1878) and *Trigona corvina* (Cockerell 1913), in coffee dominated landscapes in Southern Mexico. Acta Zoológica Mexicana 31(1): 74-79.

Sondaar, P.Y. 1977. Insularity and its effect on mammal evolution. Major patterns in vertebrate evolution (ed. by M.K. Hecht, P.C. Goody and B.M Hecht), pp. 671–707. Plenum, New York.

Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Tajima F. and M. Nei. 1984. Estimation of Evolutionary distance between nucleotides sequences. Molecular Biology and Evolution 1: 269-285.

.