Stingless bee *Tetragonula laeviceps* and *T*. aff. *biroi*: geometric morphometry analysis of wing venation variations

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Abstract. *Tetragonula laeviceps* from Pandeglang West Java is characterized by a thin honey pot. The purpose of this study was to analyze variations of wing venation among stingless bee *T. laeviceps*, *T. laeviceps* (thin honey pot) and *T*. aff. *biroi* using geometric morphometric analysis. The research was conducted by digitized the landmark at nine homologs venations of stingless bee anterior right-wing venation from 20 individuals of each species. The results show variations on wing venation in the intra- and interspecies of stingless bees based on the relative warp ordination plot. Landmark numbers 4 and number 6 are the characteristics that separate *T. laeviceps* and *T. laeviceps* (thin honey pot), and landmark numbers 1, 2, 5, and number 9 are characteristic between *T. laeviceps* and *T*. aff. *biroi*. Based on relative contribution value, landmark number 7 revealed as significant characters in the genus level and landmark number 9 are characteristics in the intraspecies of *Tetragonula*. The number of landmark variations and bending energy value of intraspecies are less compare to the interspecies of *Tetragonula*. The unrooted phylogenetic analysis shows the *T. laeviceps*, *T. laeviceps* (thin honey pot) and *T*. aff. *biroi* are separate in a different cluster for the intra- and interspecies.

Keywords: deformation grid, landmark, ordination plot, relative warp, *Tetragonula*

1. Introduction

Stingless bee (Hymenoptera: Apidae) is one of three types of eusocial bees besides Apinae and Bombinae species which are known as the main pollinators [1]. The diversity of stingless bee species is very high, approximately 600 species have been identified and described from around 61 genera [1-7]. The distribution of stingless bees is wide across the Neotropical, Afro-Tropical, Indo-Malayan and Australasian regions [1]. The high stingless bee diversity has the potential for cryptic species, that is two or more species are classified as a single species because they are morphologically indistinguishable [8].

Pollinator insects play an important role in increasing the economics and health of crops such as chili (*Capsicum annuum* L.) that were assisted by *Tetragonula laeviceps* and *T. minangkabau*. The bees effectively increase the number and weight of fruit and the number of seeds compared to chili production whose pollination is aided by the wind [9]. Moreover, stingless bees produce propolis which is an active ingredient as anti-microbial, anti-fungal, anti-oxidant and anti-inflammatory [10].

Stingless bee identification is usually conducted by morphological characters based on body part measurements. However, the identification of stingless bees, particularly in *Tetragonula* genus, encountered difficulties due to the similar characters of body size, the proportion of body color,
composition of hairs on body parts and ratio measurements in the hind leg [11]. Besides morphological characters, in certain species, nest characteristics are important to assist species identification as well.

The nest of stingless bees consists of an entrance, brood cells, storage pot (honey and pollen pot), involucrum, and batumen layer [1]. Storage pots are usually dark brown color with honey and pollen are located in separate pots. Morphological suspect T. laeviceps obtained from bee farmers in Rangkas, Banten Province has a thinner wall of honey pots. Thus, an alternative method is needed to examine the differences of Tetragonula species.

Venation of insect wings is often used as a marker of special characteristics in insects, such as stingless bee has synapomorphic features of the reduction in the venation of wings [12]. The main characteristic of stingless bee wing venation is the presence of reduced parts at marginal cells that are open apically or distal venation are narrower than region near the stigma [1]. The wing of insects has been used widely in geometric morphometry studies due to landmarks at each wing venation point and can distinguish major variations on the discriminant analysis [13].

Morphometrics describes the differences in form, variation in shape, group differences based on form, and association of forms with extrinsic factors [14] that can be applied in phylogenetic analysis. Morphometric variables based on selected landmarks represent the homologous part at the putative structure-level for use in the phylogenetic analysis[15].

Wing morphometrics explains species complexity and differences in venation between species, as studies conducted in bumble bees [16], and several stingless T. iridipennis, T. laeviceps, and L. ventralis var. arcifera [17]. The purpose of this study was aimed to analyze variations of wings venation on T. laeviceps, T. laeviceps (thin honey pot), and T. aff. biroi using geometric morphometry analysis and study the relationship among these stingless bee species.

2. Materials and methods

2.1. Stingless bee sample locations and nine homologous wing venation landmarks
Twenty samples of the right anterior wing of T. laeviceps, T. laeviceps (thin honey pot), and T. aff biroi collection of Dr. Rika Raffiudin (table 1) were used in this study with ten samples of Apis dorsata right anterior wing as outgroup. The right anterior wing was chosen due to standard in wing morphometric analysis [12, 17, 16]. The right-wing was cut at the tegulae using a scalpel or tweezers, subsequently, the wings are placed on the petri dish for photo captured Optilab camera (Olympus). Photographs of bee wings were digitized for the nine landmarks using tpsDig2 software, ordinate Cartesian were analyzed using tpsRelw, tpsSplin, and R Program [18-21]. The anatomical points of the wing were based on nine homologous points (figure 1, table 2).

| No | Species | Location | Collector| Amount |
|----|---------|----------|---------|--------|
| 1  | T. laeviceps | Cibereum Petir Village, Bogor, West Java | RR | 20 |
| 2  | T. laeviceps (thin honey pot) | Leles, Rangkas | RR, TS, YA, AP, SYW, and SB | 20 |
| 3  | T. aff. biroi | Mappedeceng Village, North Luwu, South Sulawesi | RR and TS | 20 |

*Collector: RR: Rika Raffiudin, TS: Tiara Sayusti, YA: Yofian Anaktototy, AP: Agung Prasetyo, SYW: Sri Yuliasih Wiyati, SB: Sri Bening.*
Figure 1. Venation anatomical points of the *Tetragonula* wing. *T. laeviceps* (A), *T. laeviceps* (thin honey pot) (B), and *T. aff. biroi* (C).

Table 2. Layout and description of anatomical points of the *Tetragonula* wing venation [1]

| No | Landmark   | Description                      |
|----|------------|----------------------------------|
| 1  | 1          | Point Cu – V                      |
| 2  | 2          | Point M + Cu                      |
| 3  | 3          | Point Rs + M                      |
| 4  | 4          | Meeting point of Rs and Prestigma|
| 5  | 5          | Meeting point of r & stigma        |
| 6  | 6          | Point 1m-cu 2                     |
| 7  | 7          | Point 1m-cu 1                     |
| 8  | 8          | Meeting point of Cu1 & Cu2        |
| 9  | 9          | Meeting point of V & Cu2          |

Description: 1m-cu: first medio-cubital cross-vena, Cu: cubitus, Cu1: first branch of cubitus, Cu2: second branch of cubitus, M: media (vena basal), R: radial cross-vena, r: radius, R1: first branch from radius, Rs: sector radial.

2.2. Honey pot structures
Honey pots are documented from bee farmers in Rangkas, Banten Province. The structure of the honey pot is observed by opening the *Tetragonula* nest and documenting the characteristics of the honey pot (figure 2). Honey pots are oval and slightly larger compared to pollen pots [22].

2.3. Data analysis
The stingless bee right anterior wing is analyzed using several Thin Plate Spline (TPS) software. Before analyzed, each stingless bee wing image was digitized by the *tpsDig2* version 2.31 program [19] to place the coordinates of anatomical points on wing venations. Nine anatomical venation wing landmarks (n= 5) were used in the digitizing process. The average Cartesian coordinate value of each repetition is calculated by *tpsRelw* version 1.69 program [20] resulted of two grids compose of sequence 1 is the average deformation of three *Tetragonula* species and sequence 2 derive from each species observed. Quantitative data on the deformation point is in the form of Cartesian coordinates of the x and y-axis. All software used is available at (http://life.bio.sunysb.edu/morph/).

Wing venation coordinate database for each stingless bee species was calculated based on the coordinates of each individual for each species by using the *tpsRelw* software [20]. Shape visualization of the wing deformation of each stingless bee species was used *tpsSplot* software to examine the variations in each point in an affine form [18]. Unrooted Neighbor-Joining Tree of species is made by *R* program [21] to analyze the relationship among the stingless bees.
3. Result

3.1. Variations in wing landmarks and transformation in shape among stingless bee species

Morphometric results for the stingless bee wing deformation were obtained from an analysis of 20 specimens of each species using tpsDig2 software. The results of the shape transformation due to the landmarks variations is the basic main strengths and objectives in geometric morphometrics principles as seen in Slice [14]. The venation grid deformation of the stingless bee wing illustrates the shape transformation of the tested wing samples (figure 3). The variance of landmarks numbers 4 and 6 were $S^2 = 0.00009757$ and 0.00010071, respectively which generally have higher variance value compared to other landmark points. The variance values Landmarks 8 and 3 were the two most low in variance values, i.e.: $S^2 = 0.00007176$ and 0.00008245, respectively.

Shape differences and variance in wing landmarks among species can be determined based on the difference of landmark points on the deformation grid. Variations in wing landmarks and the difference in shape among three stingless bee species were observed based on the difference of landmark on the deformation grid (figure 3). Sequence 1 was an average deformation shape of three Tetragonula species, and sequence 2 was a deformation shape of each species observed. The difference between T. laeviceps compared to the average shape of all species landmarks was in the landmark points 1, 4, and 6 which points more moving towards in, while landmark 2 moving towards lateral right from the average form (figure 3a). T. laeviceps (thin honey pots) differ from the average shape of all species in landmarks 5 and 9 which point leads inward, in contrast to landmarks 1, 2, 4, and 6 which move outward (figure 3b). All the landmarks of T. aff. biroi moving towards lateral left (figure 3c). In general, the distinct deformations occur among species observed.

We also examined the specific landmark that characterized in the genus level between the Tetragonula and Apis, shown by the high value of the relative contribution of the formation of a consensus point (table 3). Significant points characterizes genus differences (largest to the smallest contribution values) are the landmarks 2, 1, 8, and 7 and the landmarks 8, 2, 1 and 9 characterized the species differences (in order largest to the smallest values). Characteristically, landmark 7 is a genus marker and landmark 9 is characterize marker in species level of Tetragonula.
Figure 3. Wing venation and grid deformation of *Tetragonula. T. laeviceps* (A), *T. laeviceps* (thin honey pot) (B), and *T. aff. biroi* (C): Grid sequence 1, : Grid sequence 2, : Landmark sequence 1 (average deformation of all species) : Landmark sequence 2 (deformation of observed species).

Table 3. The relative contribution landmarks between stingless bee and *Apis dorsata* and among 3 stingless bee species

| No | Landmark | Stingless bee and *Apis dorsata* | Among 3 stingless bee species |
|----|----------|-------------------------------|-------------------------------|
|    |          | SS                            | SS                            |
| 1  | 1        | 0.25344*                      | 0.19977*                      |
| 2  | 2        | 0.29985*                      | 0.20937*                      |
| 3  | 3        | 0.03527                       | 0.04880                       |
| 4  | 4        | 0.04327                       | 0.04525                       |
| 5  | 5        | 0.01275                       | 0.01265                       |
| 6  | 6        | 0.04429                       | 0.02884                       |
| 7  | 7        | 0.08893*                      | 0.06936                       |
| 8  | 8        | 0.14900*                      | 0.24387*                      |
| 9  | 9        | 0.07321                       | 0.14209*                      |

* indicates the most contribution of landmarks values that have high variations
3.2. Relative warps
The average results of digitization of all samples were analyzed using tpsRelw software and consensus data, partial warps, and Relative Warp (RW) data were obtained. A total of 14 RW values were obtained from the analysis results. Visualization of the two highest values namely RW1 (22.78%) and RW2 (18.22%) is shown by the ordination plot. Based on the ordination plot, although T. laeviceps and T. laeviceps (thin honey pots) are in the same species, they can be distinguished. (figure 4).

![Figure 4. Plot ordination of RW1 and RW2 T. laeviceps, T. laeviceps (PMT), and T. aff. biroi. □ = T. laeviceps, △ = T. laeviceps (PMT), ○= T. aff. biroi.](image)

3.3. Phylogenetic tree
The next analysis is looking at the relationship of phylogeny among Tetragonula species. Euclidean distance matrix analysis was conducted to find out groupings based on similarities among the Tetragonula wings by processing data scores from the tpsRelw software. The results of the phylogeny analysis showed that each type of Tetragonula grouped in the different clusters (figure 5). Outliers are in T. laeviceps (3 individuals) and T. laeviceps (thin honey pots) (26, 29, and 38 individuals).

![Figure 5. Unrooted Neighbor Joining Tree Tetragonula. T. laeviceps (1-20), T. laeviceps (Thin honey pot) (21-40), and T. aff. biroi (41-60).](image)
4. Discussion
In addition to the cryptic potential of the species, the main obstacle in the classification of *Tetragonula* is the absence of typical structural characters in the morphology of the working caste *Tetragonula* [23]. The identification process of *Tetragonula* is based on size, proportion, color, and hair on the body and legs so that identification of certain specimens is difficult or often cannot be identified [23]. Therefore, analysis of variations in venation of wings and studied the relationship between stingless species of bees on *T. laeviceps*, *T. laeviceps* (thin honey pots), and *T. aff. biroi* was the purpose of this study.

The shape of wings and venation can be explained through homologous anatomical points. The results of the analysis show landmark 7 characterize genus and landmark 9 characterizes species. The variation of coordinates formed by landmarks not only describes the shape and size difference between specimens but also encodes the location and orientation of each specimen concerning the digital axis used to record coordinates [14]. The values of RW1 and RW2 represent the largest percentage of all possible comparisons even though they only cover 41% of all data. There are 59% of data describing other variations not taken into account in the visualization of ordinated and unrooted Neighbor-Joining plots.

This shows an interspecies comparison between *T. laeviceps* and *T. aff. biroi* having low wing shape variations. A comparison of intraspecies between *T. laeviceps* and *T. laeviceps* (thin honey pots) indicates the presence of closeness by grouping and adjacent points on the ordination plot. This indicates the similarity in morphology and venation of the wings between the two. However, these variations can still illustrate interspecies differences seen from the ordinated and unrooted Neighbor-Joining plots that cluster in each species. This is consistent with the research conducted by Vijayakumar and Jayaraj [17] which states that the morphometric geometry method can distinguish clearly among *T. iridipenis*, *T. laeviceps*, and *Lepidotrigona ventralis* var. *arcifera* [17]. The relative position of the landmark which contributes most of the Cartesian coordinate is the configuration of 'x' in landmarks 3,4,5,6 and 9 and 'y' in landmarks 1,2,7,8 and 9 [17]. Or in other words landmarks, number 9 is the most contributing point to the configuration of 'x' and 'y'.

*Tetragonula* is a type of stingless bee that builds a cluster nest-type with homomorphic pots of pollen and honey [24]. The difference in nest shape may be related to the phenomenon of polyphenism in insects (figure 2). Polyphenism is an adaptive response to the environment. Polyphenism is the key for insects at every stage of their lives to produce the best phenotype in the face of predictable environmental changes [25]. Insects express different optimal phenotypes for each environment that they selectively meet and individuals will maximize appropriate morphological expressions in appropriate circumstances [26].

This study used a sample of stingless bee wing worker castes as the object under study. Holometabola insect phenotype can be seen not only based on morphology but also based on behavior, such as reproductive behavior and specialization behavior for specific tasks related to the maintenance of colonies [26]. Differences in the shape of the nest on *T. laeviceps* and *T. laeviceps* (thin honey pots) may be different forms of phenotype although morphologically are not different from each other. This can be an indication that changes are happening towards the speciation process that occurs in a long and periodic process.

Overlap or outliers in individuals 3, 26, 29, and 38 besides being caused by species similarity, may also occur due to several factors. Polyphenism allows overlap in species because of their adaptation to the environment. Polyphenism drive morphological changes including morphological traits and morphological properties influenced by the environment which can obscure differences between species [27].

The results of this study indicate that geometric morphometric methods can see the difference between *T. laeviceps*, *T. laeviceps* (thin honey pot), and *T. biroi* based on anatomical points on the wings. The analysis using the geometric morphometric methods showed differences between *T. laeviceps* and *T. laeviceps* (thin honey pot) although they cannot be distinguished morphologically.
Geometric morphometrics can also see potential and influential points in characterizing genus and species. Thus, geometric morphometric can be used for identifying species of stingless bees.

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