

Discrimination of Two *Tetragonula* (Apidae: Meliponini) Species in Thailand using Geometric Morphometric Analysis of Wing Venation

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ABSTRACT

Geometric morphometrics is a novel powerful tool for the identification of bee species and subspecies. An application of this method is presented to discriminate two stingless bee species, *Tetragonula pagdeni* Schwarz, 1939 and *Tetragonula fuscobalteata* Cameron, 1908, in Thailand based on forewing venation. Nine landmarks were measured from the digitized right forewing of 200 workers (10 bees per colony). Eleven colonies of *T. pagdeni* and nine colonies of *T. fuscobalteata* collected from several locations in Thailand were determined. The positions of the groups in the principal component analysis plots revealed that the species are clearly distinct. Multivariate analysis of variance ($\alpha = 0.01$) showed a significant difference between the bees of the sibling species, producing a high degree of accuracy (96.50%) in the identification of any individual bee of either species. Therefore, it is proposed that geometric morphometric analysis of single wings might be an appropriate first step in the analysis and identification of stingless bees and other insects.

Keywords: stingless bees, geometric morphometrics, wing venation pattern, *Tetragonula pagdeni*, *Tetragonula fuscobalteata*

INTRODUCTION

The importance of stingless bees as effective pollinators of many plant species in natural and agro-ecosystems has been well documented (Heard, 1999; Amano *et al.*, 2000; Slaa *et al.*, 2000; Biesmeijer *et al.*, 2006; Klein *et al.*, 2007; Quezada-Euán *et al.*, 2012), and they seem to be good candidates for applications as commercial pollinators (Slaa *et al.*, 2006; Theeraapisakkun *et al.*, 2010). However, there is increasing evidence

of declines in stingless bee populations (Potts *et al.*, 2010; Francoy *et al.*, 2011), mainly affected by habitat deterioration (Biesmeijer *et al.*, 2006; Winfree *et al.*, 2009; Williams *et al.*, 2010). Thus, the accurate identification of stingless bee species is urgently needed to investigate their biodiversity (Francoy *et al.*, 2009; May-Itzá *et al.*, 2012).

Stingless bees (Meliponini) are one of the most morphologically and behaviorally abundant and diverse species among the corbiculate bees (Apini, Bombini and Meliponini) (Michener,

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2007; Rasmussen and Cameron, 2010). Unlike the Apini, with only 11 species in the single genus *Apis* (Lo *et al.*, 2010), stingless bees are a large and diverse taxon of more than 400 species comprising some 60 genera (Michener, 2007; Moure *et al.*, 2007), many of which are poorly known (Rasmussen and Cameron, 2010). In some cases, these diverse species suggest the possible existence of many species complexes in the Meliponini (Michener, 2007; May-Itzá *et al.*, 2012). Thus, the identification of stingless bee species remains difficult and requires analysis by an experienced taxonomist (Dollin *et al.*, 1997; Theeraapisakkun *et al.*, 2010).

Various molecular methods, including analyses of mitochondrial and nuclear DNA sequence polymorphism, have been widely used to discriminate among bee species and subspecies (Francisco *et al.*, 2001; Whitfield *et al.*, 2006; Ramirez *et al.*, 2010; Rasmussen and Cameron, 2010; Theeraapisakkun *et al.*, 2010; Quezada-Euán *et al.*, 2012). However, these methods require relatively expensive reagents, specialized personal knowledge and a well-equipped laboratory (Francoy *et al.*, 2008).

Traditional morphometric analysis, which is based on multiple measurements of various body parts across many individuals, has recently become widely used for identifying bee species and subspecies (Daly *et al.*, 1982; Chaiyawong *et al.*, 2004; Radloff *et al.*, 2005; Francoy *et al.*, 2006; Rattanawanee *et al.*, 2007; Andere *et al.*, 2008; Özkan *et al.*, 2009), because of its high practicability and low cost (Francoy *et al.*, 2008). However, traditional morphometric analysis requires time-consuming specimen preparation and measurement procedures (Francoy *et al.*, 2006). Moreover, the complex shape of an organism cannot easily be summarized by using linear measurements as in traditional morphometrics (Pavlinov, 2001; Aytekin *et al.*, 2007).

Instead of distance and angle measurements, geometric morphometrics uses

coordinates of homologous landmarks, which offers superior discrimination capabilities to traditional morphometry (Bookstein, 1997; Azidah *et al.*, 2000; Villemant *et al.*, 2007). Importantly, geometric morphometric analysis can exclude the effects of size as it uses the relative positions of the plotted landmarks. Therefore, environmental influences, for instance nutrition and parasitism, can be reduced or removed (Villemant *et al.*, 2007; Francoy *et al.*, 2011).

The geometric morphometric analysis of wings has been applied for the identification of bee specimens to genera, species, subspecies and even populations (Mendes *et al.*, 2007; Francoy *et al.*, 2008, 2011; Kandemir *et al.*, 2009). For example, Francoy *et al.* (2006) showed that a single wing cell carries enough information for the identification of the three racial groups of *Apis mellifera* (Africanized, Italian and Carniolan) with a fidelity level of nearly 99% of the individuals. Francisco *et al.* (2008) showed that a geometric morphometric analysis of wing venation can be used to resolve the taxonomic identification of cryptic species in *Plebeia remota*. Francoy *et al.* (2009) reported that landmarks based on the geometric morphometric analysis of the wing shape of males and workers were sufficient to discriminate among five stingless bee species and that the sex of individuals does not impede identification. Moreover, Villemant *et al.* (2007) also reported that applying geometric morphometrics to wing venation could resolve a complex case of four sibling parasitoid wasp species in the genus *Eubazus* (Hymenoptera, Braconidae).

Even though geometric morphometric analysis is a simple methodology for which only a stereomicroscope, a digital camera and a computer are needed (Francoy *et al.*, 2011), it gives clear results to answer the questions that are normally resolved using molecular methods (Francoy *et al.*, 2008, 2011).

Stingless bees in the genus *Tetragonula* comprise the largest and most widespread group in the Indopacific area, including some of the

commonest species (Sakagami, 1978). Some stingless bee species are easily distinguished from others. But some species require critical studies, especially those that appear to be cryptic species as is the case for *Tetragonula pagdeni* and *Tetragonula fuscobalteata*. They are very difficult to distinguish because of the great overlap in size so that traditional morphometrics are inadequate to identify them. Therefore, the objective of this study was to apply a geomorphometric method using the characteristics of the forewings of workers to identify two sibling stingless bee species in Thailand, *T. pagdeni* Schwarz, 1939 and *T. fuscobalteata* Cameron, 1908 (Sakagami and Khoo, 1987; Theeraapisakkun *et al.*, 2010). The identification accuracy is discussed and whether or not employing such geometric morphological

techniques will be an alternative method with greater accuracy and relevance to distinguish between these two taxonomically problematic species.

MATERIAL AND METHODS

Sample collection sample preparation and measurement

Adult workers of 11 colonies from 8 locations of *T. pagdeni* and 9 colonies from 6 locations of *T. fuscobalteata* were sampled (Table 1, Figure 1). At least 30 workers from each colony of the two species were collected directly at the nest entrance of each colony and stored in 75% ethanol. Ten workers from each colony were randomly selected for dissection. The

Table 1 Collection of two stingless bee species from Thailand.

No.	Sampling location	Coordinates	<i>T. pagdeni</i>	<i>T. fuscobalteata</i>
1	Chiang Mai	18° 53' 17.16'' N 98° 51' 41.96'' E	1 colony	1 colony
2	Maha Sarakham	16° 12' 09.42'' N 103° 16' 59.27'' E	1 colony	-
3	Nakhon Sawan	16° 00' 36.88'' N 99° 54' 58.70'' E	-	1 colony
4	Nakhon Sawan	15° 58' 37.01'' N 99° 54' 17.23'' E	-	1 colony
5	Bangkok	13° 44' 13.79'' N 100° 31' 48.40'' E	1 colony	-
6	Chanthaburi	12° 30' 43.99'' N 102° 10' 11.40'' E	1 colony	2 colonies
7	Ratchaburi	13° 35' 24.73'' N 99° 30' 27.12'' E	3 colonies	3 colonies
8	Samut Songkhram	13° 23' 13.49'' N 99° 57' 11.59'' E	2 colonies	-
9	Chumphon	10° 29' 46.75'' N 99° 08' 47.03'' E	-	1 colony
10	Chumphon	10° 30' 00.11'' N 99° 09' 42.59'' E	1 colony	-
11	Chumphon	10° 28' 46.85'' N 99° 12' 01.68'' E	1 colony	-
Total			11 colonies	9 colonies

right forewing of each bee sample was removed, slide-mounted and photographed with a digital camera on a stereomicroscope. From a total of 200 wings, 110 wings of *T. pagdeni* workers and 90 wings of *T. fuscobalteata* workers were measured. Nine homologous landmarks, which are the most easy observable and often adopted for insect identification, on the intersections of forewing veins were manually plotted (Figure 2) using the tpsDig2 version 2.16 software (Rolhf, 2005a). The Cartesian coordinates of the landmarks were then Procrustes aligned and a partial warps analysis was performed using the tpsRelw version 1.49 software (Rolhf, 2005b). The mean configuration of the bees from a colony was used as a comparative parameter and the analysis was made at the colony level (Francisco *et al.*, 2008; Francoy *et al.*, 2011).

Data analysis

A principal component analysis (PCA) was performed using the Cartesian coordinates of each homologous landmark after alignment (Amssalu *et al.*, 2004; Francoy *et al.*, 2006, 2009, 2011). A stepwise analysis was carried out to determine classification functions, followed by a canonical analysis. Then a cross-validation test was performed on 10% of randomly selected individuals as unknowns (Francisco *et al.*, 2008; Rattanawanee *et al.*, 2010, 2012), and the data used to calculate and check the accuracy of the equations in identifying the colonies (Francoy *et al.*, 2011). The square Mahalanobis distances were also calculated between the centroids of the bee sample groups (Francoy *et al.*, 2009; Francoy *et al.*, 2011). After extraction of the measures, all statistical analyses were performed using SPSS

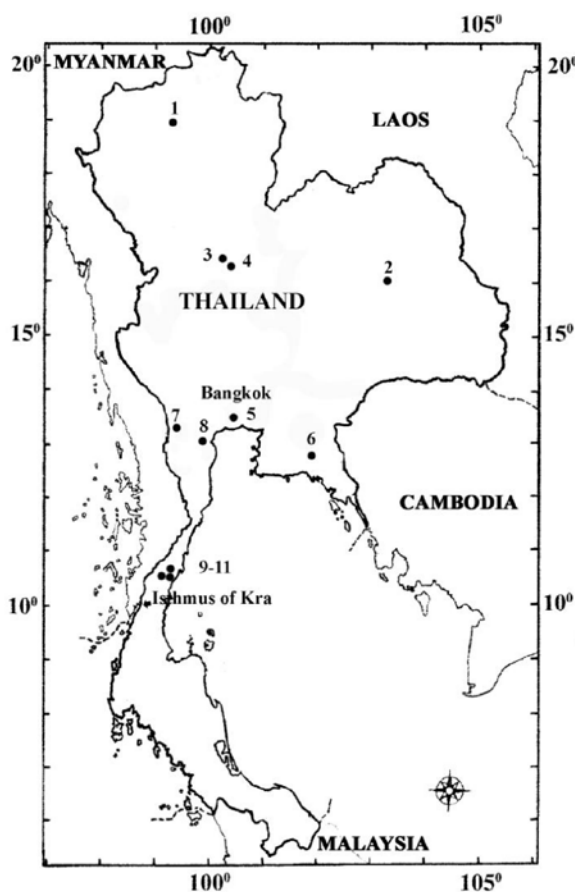


Figure 1 Two stingless bee species collection sites in Thailand. Numbers correspond to those in Table 1

Version 10.0 (1999; SPSS; Chicago, IL, USA).

RESULTS

The nine anatomical landmarks (Figure 2) generated 14 relative warp measurements for each of the wing specimens. The four highest relative warps explained 71.96% of the group's variability, with 38.03%, 12.63%, 11.58% and 9.72% being explained by the first, second, third and fourth relative warps, respectively.

Five principal factors with eigenvalues greater than one were extracted from the PCA analyses. Together, the five components were able to explain 68.07% of the data set variability. The first two main principal components explained 41.06% of the variability. The coordinate variable 7x mostly influenced the first factor and explained 25.22% of the variability among the groups, whereas the coordinate variable 1y mostly influenced the second factor and explained 15.85% of the variation.

The positions of the groups in the PCA plots of factor 1 (25.22%) and factor 2 (15.85%), show that the species groups are well distinguished (Figure 3), with the *T. pagdeni* samples placed mainly in the upper left-hand quadrant and *T. fuscobalteata* samples mainly in the lower right-hand quadrant of the plot so that these two

sibling species of *Tetragonula* species are clearly separated (Figure 3).

Multivariate analysis of variance of the measures demonstrated that the stingless bee groups were significantly different (Wilk's $\lambda = 0.209$; $P < .001$). The linear discriminant analysis of the data showed that the centroid sizes of the groups were significantly different ($P < .001$) from each other.

From the 200 wings, seven were misclassified in the respective group (Figure 4), five from *T. pagdeni* and two from *T. fuscobalteata*. The cross-validation test using 10% of the individuals as unknowns correctly identified 96.5% of the individuals to each respective group.

DISCUSSION

In view of the fact that the two species, *T. pagdeni* and *T. fuscobalteata* have always been difficult to identify, to achieve this end was a first priority. Inasmuch as the bees can now be clearly separated, it is only as of now possible to pursue information on the basic biological differences between them and, ultimately, to develop suitable management techniques for their future use in Thailand.

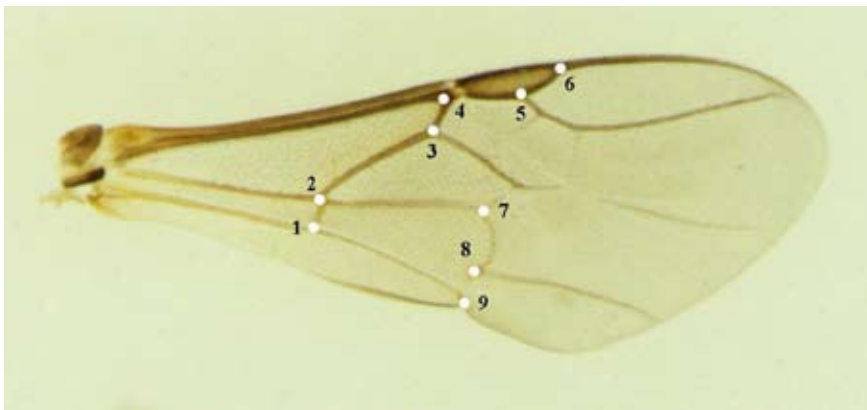


Figure 2 Right forewing of a *T. pagdeni* worker. The white circles indicate the respective position of each of the plotted landmarks.

In recent years, the geometric morphometric analysis of wings has been used to discriminate hymenopteran species, subspecies and populations including wasps (Baylac *et al.*, 2003; Villemant *et al.*, 2007; Abbasi, 2009), bumble bees (Francoy *et al.*, 2006, 2008; Aytekin *et al.*, 2007), honey bees (Andere *et al.*, 2008;

Tofilski, 2008; Rattanawanee *et al.*, 2010, 2012;) and stingless bees (Mendes *et al.*, 2007; Francisco *et al.*, 2008; Francoy *et al.*, 2009, 2011). Tofilski *et al.* (2008) showed that the geometric analysis of forewing venation is a more powerful tool than standard morphometric analysis (using the distance and angle of various parts of the

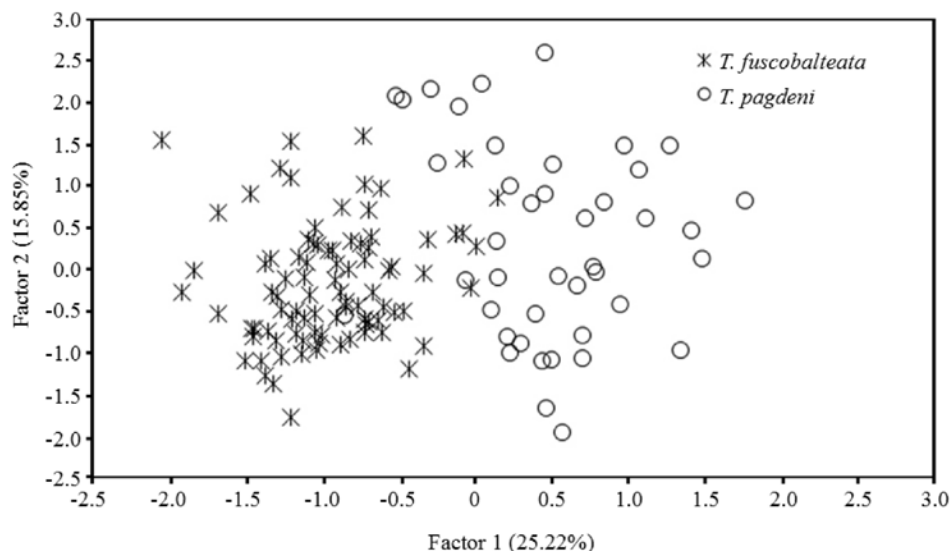


Figure 3 Scatterplot of the two most influential factors (variable 7x and 1y, respectively) from principal component analysis of workers of two stingless bee species (*Tetragonula pagdeni* and *T. fuscobalteata*) measured for nine landmarks.

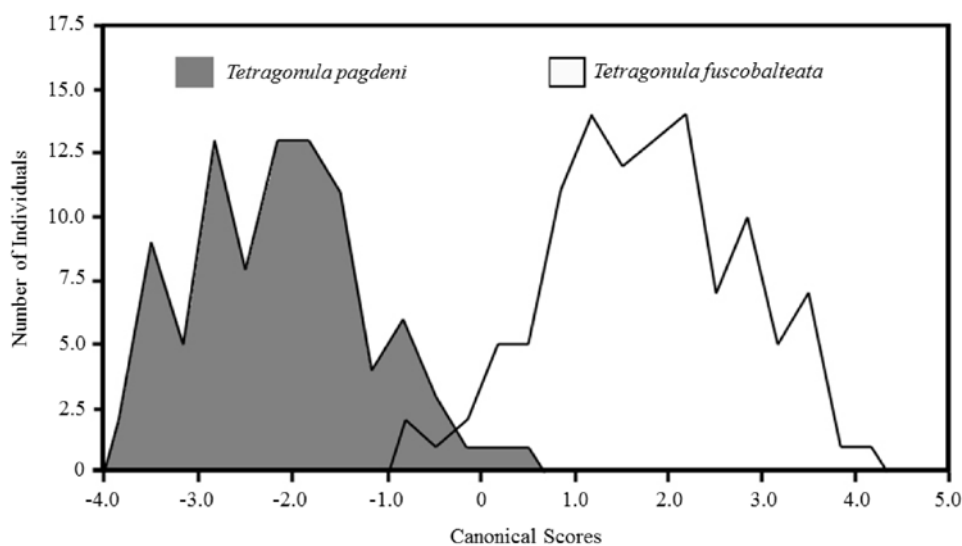


Figure 4 Canonical distributions of the individuals from two stingless bee species, *Tetragonula pagdeni* and *T. fuscobalteata*, based upon measurements extracted from forewing venation pattern.

body) for the discrimination of subspecies of *A. mellifera*. Furthermore, Francoy *et al.* (2011) demonstrated that the geometric morphometry of the wing venation is sensitive enough to be used for discrimination between Mexican and Central American populations of *Melipona beecheii*. This result was supported by a study of internal transcribed spacer (ITS) ribosomal DNA sequence-based analysis, which revealed that *M. beecheii* from Mexico presents an ITS1 haplotype (ITS1-A) different from the bees from Central America (ITS1-B and ITS1-C) (May-Itzá *et al.*, 2012).

Sibling species are expected to reveal high morphological similarity (Aytekin *et al.*, 2007); however, some morphological differences that allow them to be distinguished can be found when morphometric analysis is used (Moraes *et al.*, 2004; Aytekin *et al.*, 2007;).

Among 32 stingless bee species that have been recorded in Thailand (Schwarz, 1939; Sakagami *et al.*, 1985; Michener and Boongird, 2004; Klakasikorn *et al.*, 2005), some sibling species such as *T. pagdini*, *T. laeviceps*, and *T. fuscobalteata* are sympatric species which often complicates any preliminary identification based on the geographic distribution and basic morphological characteristics (Sakagami and Khoo, 1987; Starr and Sakagami, 1987). Even though the species diagnostic CUTP1 SCAR marker for identification has been successfully developed for *T. pagdeni* (Thummajitsakul *et al.*, 2010), the limit of technical skill and cost in species identification between species still remain. The geometric morphometric analysis performed in the current study on *T. pagdeni* and *T. fuscobalteata* demonstrated that this method is efficient for correctly assigning individual colonies to their species. The results demonstrated that the forewing shapes show significant differences between these two stingless bee species, as has also been found for bumblebees (Aytekin *et al.*, 2007), wasps (Villemant *et al.*, 2007), Diptera (Moraes *et al.*, 2004) and Hemiptera (Gumieli *et al.*, 2003).

The current results are in close agreement with those of Francoy *et al.* (2009) who showed that wing venation patterns alone are sufficient to classify five sibling species of stingless bees. They made geometric morphometric analyses of the forewings of male and workers of *Nannotrigona testaceicornis*, *Melipona quadrifasciata*, *Frieseomelitta varia*, *Scaptotrigona depilis*, and *Plebeia remota* and showed that the patterns of males and workers from the same species were more similar than the patterns of individuals of the same sex from different species. The origins of the wing shape differences are unclear at present (Aytekin *et al.*, 2007; Francoy *et al.*, 2009). In the case of stingless bees, there can be some adaptive constraints such as foraging behavior, mating behavior, flying mechanisms and pollen loads (Aytekin *et al.*, 2007).

The current study was able to discriminate between two stingless bee species based on wing information using a digitized wing image, which greatly facilitated and accelerated analysis. It was concluded that measurements of a small part of the entire bee body are sufficient to discriminate among stingless bee species. This methodology is simple and could be extended to finer identifications among species of bees with the addition of future landmarks. Moreover, computer programs to assist in the morphometric analysis of the wing might be a useful implementation in biodiversity and conservation studies.

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