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Wing morphometrics indicates the existence of two distinct phenotypic clusters within population of *Tetragonula iridipennis* (Apidae: Meliponini) from India

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Abstract Description of biodiversity is often cited as one of the most important actions necessary for conservation programs. There are more than 600 species of stingless bees spread over the Tropical regions of the world; though for various species, little is known about their biology and taxonomy. We sampled bees from feral colonies from various regions of India and compared them using wing morphology. The results of population analysis of the patterns of wing venation, using geometric morphometric techniques, suggested the existence of at least two phenotypic clusters within our samples of the so-called *Tetragonula iridipennis* complex. These findings were supported by other features, including differences in nest architecture. This helps to explain the patterns of variability found in stingless bees in India and also will be valuable for conservation planning.

Keywords *Tetragonula iridipennis* · Geometric morphometrics · Wings · Biodiversity · Stingless bees · Conservation

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Introduction

Stingless bees (Apidae, Apinae, Meliponini) are closely related to the other corbiculate bees, including honey bees, orchid bees and bumble bees (Roubik 1989; Kawakita et al. 2008; Danforth et al. 2013; Hedtke et al. 2013). The presence of these bees in New Jersey amber from the Late Cretaceous suggests that these are amongst the earliest evolved bees and that they developed before the continents drifted apart (Engel 2000). In fact, they are present in most tropical and subtropical regions of the world. Stingless bees are a large and diverse group, comprising about 60 genera and more than 600 species, many of which are poorly known (Rasmussen and Cameron 2010).

The Meliponini are highly eusocial, very diverse in size and colony population, with a wide range of nesting and feeding behaviors that allow them to share habitats, resulting in high colony densities in nature. They are a widely diversified group, occupying the pan-tropical region of the world (Roubik 1989; Michener 2000). These bees are valuable pollinators of wild plants, as well as agricultural, horticultural and plantation crops. Heard (1999) reviewed the importance of stingless bees as pollinators and reported that they are effective and important pollinators of nine different crops, and that they contribute to the pollination of 60 other crops. Important crops pollinated by these bees include macadamia, coconut, mango, chayote, watermelon, guava, strawberry, citrus, avocado, lychee, coffee and cardamom. *Trigona* and *Melipona* are major honey producing stingless bees, and are very well studied and have been kept in containers since the time of the Mayan culture in Mexico (Cortopassi-Laurino et al. 2006). They are also kept in hives or pots in other parts of South America, Africa, Australia and Asia, including India. Meliponine honey is prized as a medicine in these countries (Cortopassi-Laurino et al. 2006).

Due to their importance as crop pollinators and also as honey producers, numerous studies about bee conservation have been carried out throughout the world, including genetic status of populations (Batalha-Filho et al. 2010), diploid male production and colony management (Alves et al. 2011), and development of auxiliary tools for species identification (Francisco et al. 2008; Hurtado-Burillo et al. 2013). Although their taxonomy is very well known for some groups, including the genus *Melipona* (Ramírez et al. 2010), most genera still require taxonomic revision.

It was shown, based on molecular data, that the so-called *Trigona* genus is not monophyletic, demonstrating the need for taxonomic review of the group. Currently, nine species of stingless bees are known from the continental India, classified in three genera, namely *Lepidotrigona*, *Lisotrigona*, and *Tetragonula*. Most of the species are classified as belonging to the genus *Tetragonula* (Rasmussen and Cameron 2010). This group has the largest and most widespread distribution in the Indo-pacific region. Hence Moure (1961) pointed out that this group needs to be studied very critically. Accordingly, Sakagami (1978) proposed that *Tetragonula iridipennis* is confined to India and Sri Lanka; this species was formerly regarded as having a widespread distribution, from India to the Solomon Islands. Currently, although efforts are being made to clarify the taxonomy of these species, doubts still remain about the taxonomic status of Asian stingless bees, and the *Tetragonula iridipennis* complex is a clear example. Therefore, there is an urgent need to survey this group in Continental India, in order to clearly define the limits of these species (Rasmussen 2013).

The existence of cryptic species is well described in bees, and differentiation of some species is only possible when molecular (Gibbs 2009), morphometric and biochemical (Francisco et al. 2008), or behavioral features are used (Camargo and Pedro 2003). Among these methods, DNA barcodes (Hebert et al. 2003) and geometric morphometrics of wings (Francoy et al. 2012; Bonatti et al. 2014) are the most widely used and effective. Although wing venation of stingless bees is greatly reduced, geometric morphometrics of forewings has proven to be a useful tool for investigations of these bees (Francisco et al. 2008; Francoy et al. 2009, 2011). Geometric morphometrics of wings has been widely applied to taxonomic studies of various species groups, including stingless bees (Mendes et al. 2007; Francoy et al. 2006, 2008, 2009; Bonatti et al. 2014). Additionally, this technique allows geographic traceability of populations (Francoy et al. 2011), identification of origin and dispersion route of ancient European honey bee lineages (Kotthoff et al. 2013), recognition of close relatives of bee fossils (Wappler et al. 2012), as well as reassessment of stingless bee taxonomy (Dewulf et al. 2014). Geometric morphometrics of wings has also been shown to be particularly efficient in evaluating insect diversity, being useful in the

identification of species (Francoy et al. 2009), subspecies (Oleksa and Tofilski 2014), cryptic species (Francisco et al. 2008), geographic ecotypes (Francoy et al. 2011), genetic lineages (Quezada-Euán et al. 2012; Bonatti et al. 2014), and also subtle intraspecific variation due to phenotypic plasticity (Debat et al. 2003; Jorge et al. 2011; Batista et al. 2013). Although wing shape is relatively conserved among individuals of the same species, geometric morphometrics has shown to be a valuable and sensitive tool for the analysis of even very fine differentiation among groups (Debat et al. 2003).

In India, even though stingless bees have been known to be highly beneficial for centuries, there have been very few systematic studies on the taxonomy of these bees (Sakagami 1978; Rasmussen 2013; Vijayakumar and Jeyaraaj 2014), including an analysis of three species, using geometric morphometrics of wings (Vijayakumar and Jeyaraaj 2013) Hence, in order to generate pertinent data, we used wing morphometrics to examine the variability of the Indian populations of *Tetragonula iridipennis*.

Materials and methods

Workers of *Te. iridipennis* were collected from 150 locations in seven states of India, namely, Karnataka ($n = 244$), Tamil Nadu ($n = 91$), Kerala ($n = 66$), Andhra Pradesh ($n = 121$), Maharashtra ($n = 48$), Assam ($n = 5$) and Meghalaya ($n = 3$), representing different agroclimatic regions in these states (Supplementary Table 1). At each location, a thorough search was made for feral colonies of *Te. iridipennis*. Samples of bees from each colony were collected by gently tapping near the entrance, inducing workers to come out. Three to five workers from each colony were stored in vials containing 75 % ethyl alcohol. In the locations where feral colonies could not be located, bees were collected from forage resources, such as flowers and fruit, with the use of an aspirator. Representative samples were later mounted and labeled.

We also included samples of five *Trigona* species [*Trigona recursa* ($n = 118$), *Tr. hyalinata* ($n = 38$), *Tr. spinipes* ($n = 76$), *Tr. hypogea* ($n = 20$) and *Tr. fuscipennis* ($n = 30$)] collected from the campus of the University of São Paulo (Ribeirão Preto, Brazil) to compare morphological distances between these described Brazilian species and the Indian populations.

The right forewing was carefully detached from the body with a forceps, and mounted between microscope slides. The 860 forewings were photographed with a Leica stereoscopic binocular microscope (model: M 165 C), using a digital camera, and the images stored as digital files.

Ten homologous landmarks were plotted at the wing vein intersections (Fig. 1), using the software tpsDig2 version
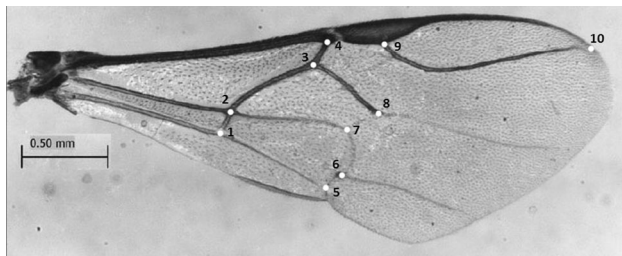
Fig. 1 Right forewing of a *Tetragonula iridipennis* worker. The *white circles* indicate the plotted landmarks and the *number* represent the order that the landmarks were marked

2.12 (Rohlf 2008). In order to compare differences among the groups, the images were Procrustes aligned using software tpsRelw version 1.45 (Rohlf 2007). The partial warps and the uniform component X and Y were extracted and used as input features in a canonical discriminant analysis using the software STATISTICA version 6.0 (StatSoft 2001). The Procrustes distances among the studied groups were calculated using MorphoJ (Klingenberg 2011). We have also performed a leave-one-out cross-validation test based on the Mahalanobis distances between individuals and centroids using STATISTICA 6.0 (Statsoft 2001). We have also calculated the mean distances between individuals and the centroids of its own group and also the mean distances of individuals to the centroids of other groups.

Bees were grouped according to the states where they were collected in India and also the five species of *Trigona* from Brazil. The Mahalanobis square distances between the centroids of the groups and the Procrustes distances between mean shapes of the groups were extracted and used in the construction of dendograms of morphological proximity, based on UPGMA distances, using MEGA 5.0 (Tamura et al. 2011). In order to verify the reliability of the constructed dendograms, we have calculated the coefficient of cophenetic correlation using the software Treefit (Kalinowsi 2009). The Procrustes distances between the groups and the distances between the centroids calculated by Mahalanobis square distances from the Neotropical species were used as parameters to compare the distances between the centroids of the Indian groups and the Procrustes distances between them.

Results

The partial warps and the two uniform components used in the canonical discriminant analysis were included in the discriminant model; overall discrimination among the groups was significant (Wilk's Lambda = 0.00012; $p < 0.0001$). The Mahalanobis square distances between the centroids of the groups were calculated (Table 1). The canonical analysis indicated that all of populations/species formed cluster that were significantly different from each other ($p < 0.05$), except for the groups of the Meghalaya and Karnataka populations ($p = 0.23$) and the Meghalaya and Assam populations ($p = 0.82$). The Procrustes distances among the groups (Table 2) showed a similar pattern. The dendogram of morphological proximity built based on the Mahalanobis square distances between the centroids of the groups (Fig. 2) placed all the species from the Neotropics in one branch and all the Indian populations in another branch. In the Neotropical branch, *Tr. hyalinata* and *Tr. spinipes* were placed in the same sub-branch, while *Tr. fuscipennis*, *Tr. hypogea* and *Tr. recursa* were placed in another sub-branch. In the Indian populations branch, the populations of Assam and Meghalaya were placed in one sub-branch and all the other populations were placed in another sub-branch. The calculated coefficient of cophenetic correlation was 0.98, indicating that the dendogram is

Table 1 Mahalanobis square distances between the centroids of the groups calculated from the partial warps and the Uniform component X and Y extracted from the landmarks plotted on the wings

	Kerala	Maharashtra	Tamil Nadu	Andhra Pradesh	Karnataka	Assam	Meghalaya	*Tr. fuscipennis*	*Tr. hyalinata*	*Tr. hypogea*	*Tr. recursa*
Maharashtra	8.88										
Tamil Nadu	6.02	6.65									
Andhra Pradesh	3.95	8.53	7.26								
Karnataka	7.08	8.37	7.32	11.24							
Assam	21.51	25.22	23.87	29.74	12.85						
Meghalaya	14.15	19.01	17.64	21.46	6.79	5.86					
Tr. fuscipennis	455.19	471.82	457.63	463.87	469.38	438.41	437.22				
Tr. hyalinata	413.06	449.32	416.77	414.87	449.74	435.19	424.62	93.25			
Tr. hypogea	348.68	373.69	359.13	358.3	367.87	338.05	336.3	43.89	75.45		
Tr. recursa	393.49	409.6	393.3	398.63	414.41	386.28	390.29	26.08	92.91	22.38	
Tr. spinipes	375.06	401.51	377.07	383.05	401.85	397.45	377.06	85.33	59.84	47.47	64.9

Table 2 Procrustes distances between the groups indicating differentiation of the mean shape of the patterns of wing venation

	Andhra Pradesh	Assam	Karnataka	Kerala	Maharashtra	Meghalaya	*Tr. fuscipennis*	*Tr. hyalinata*	*Tr. hypogea*	*Tr. recursa*	*Tr. spinipes*
Assam	0.0368										
Karnataka	0.0235	0.022									
Kerala	0.0124	0.0334	0.0211								
Maharashtra	0.0218	0.0258	0.0185	0.0246							
Meghalaya	0.0314	0.0135	0.0154	0.0276	0.0247						
Tr. fuscipennis	0.1276	0.1161	0.1224	0.129	0.1218	0.1177					
Tr. hyalinata	0.0902	0.0944	0.0946	0.0905	0.0952	0.0925	0.0754				
Tr. hypogea	0.0957	0.0894	0.0957	0.0965	0.0947	0.0902	0.0521	0.0456			
Tr. recursa	0.1179	0.111	0.1174	0.1202	0.1148	0.1128	0.037	0.0672	0.034		
Tr. spinipes	0.0901	0.0907	0.0927	0.0907	0.0915	0.0894	0.0651	0.0323	0.0297	0.0521	
Tamil Nadu	0.0158	0.029	0.0159	0.0123	0.0193	0.0242	0.1243	0.09	0.095	0.1172	0.0894
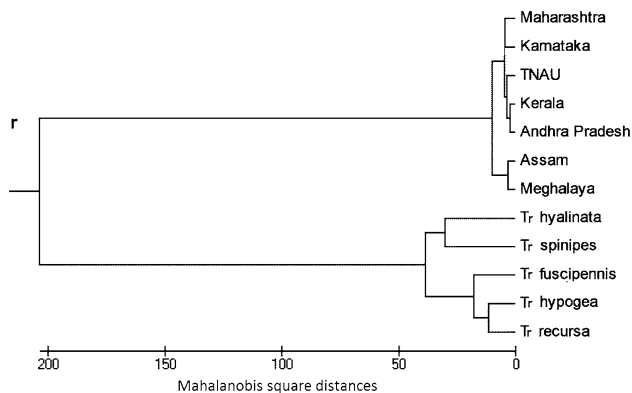
Fig. 2 UPGMA dendogram constructed based on the Mahalanobis square distances between the centroids of the groups. *TNAU* Tamil Nadu
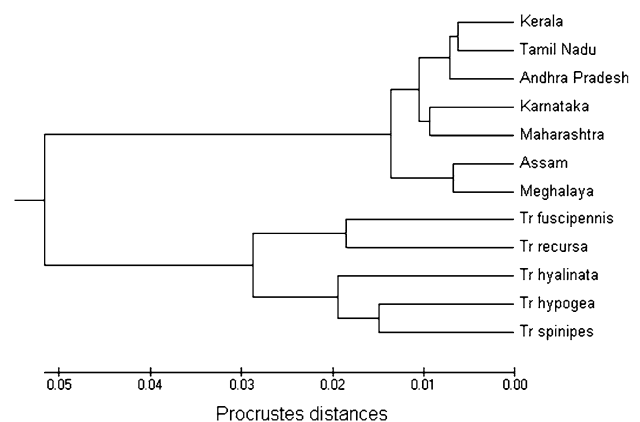
Fig. 3 UPGMA dendogram constructed based on the Procrustes distances between the groups

a good graphical representation of the calculated Mahalanobis square distances between the centroids of the groups. A similar situation was observed when the dendogram was built based on the Procrustes distances among mean shape of the groups (Fig. 3). The main structure was maintained, with the Indian populations placed in one branch and the Neotropical species in another branch. In the Indian branch, the subdivision was also maintained; the populations of Assam and Meghalaya were placed in one sub-branch and all the other populations were placed in another sub-branch.

The Mahalanobis square distances between the centroids of the Neotropical species ranged from 22.38 (between *T. hypogea* and *T. recursa*) to 93.25 (between *T. fuscipennis* and *T. hyalinata*), while in the Indian groups, they ranged from 3.95 (between Andhra Pradesh and Kerala) to 29.74 (between Assam and Andhra Pradesh). Among the Indian populations, all the distances between the centroids were smaller than 10, while the distances of the populations from

Assam and Meghalaya to all the other Indian populations were often greater than 20, close to the distances between some of the Neotropical species.

We compared the intraspecific variation of the Indian populations with the range of Mahalanobis square distances between the centroids of the groups. In the Assam and Meghalaya populations, the Mahalanobis square distances between the individuals and the centroids of the groups ranged from 2.49 to 5.37, which is shorter than the distances between the centroids of the groups. The distance between these individuals from the other Indian populations ranged from 13.12 to 20.15. In the other populations, the intra-group distances ranged from 5.87 to 13.42, comparable to the distances between these populations, and the inter-groups distances ranged from 6.14 to 18.24.

The cross-validation test correctly classified 100 % of the individuals from Assam and Meghalaya within these two groups; no individual from the other populations was identified as belonging to either of these two populations. In

contrast, the rates of correct classification for the individuals of the other populations were 47.9 % in Maharashtra, 50.0 % in Kerala, 76.9 % in Tamil Nadu, 72.7 % in Andhra Pradesh and 89.3 % in Karnataka.

The plot of the individuals in the two first discriminant axis discriminant scores (Supplementary Figure 2) showed a great superimposition of the individuals of all groups, except for the individuals from the regions of Assam and Meghalaya, which are marginal to the plot and have a superimposition only with the population of Karnataka. The individuals from Assam and Meghalaya also form a more compact group, when compared to the other groups.

Discussion

Tetragonula iridipennis is one of the eight described stingless bee species found in the Indian subcontinent; though it probably consists of several species (Rasmussen 2013). Our results suggest that there is indeed more than phenotypic cluster within our sample. This conclusion is based on the separation between the populations from Assam and Meghalaya and the other populations, using both the Mahalanobis square distances between the centroids of the groups and the Procrustes distances between the groups. They are comparable to the distances found between the five Brazilian species of *Trigona* that we examined (Figs. 2, 3; Tables 1, 2). In other comparable situations between populations of a known species and other species of the same genus, the distances between populations is always smaller than the distances between species. This is the case for *Melipona subnitida* populations in Northeast Brazil (Bonatti et al. 2014), for *Meliponula bocandei*, *Axestotrigona ferruginea*, *Dactylurina staudingeri* and *Hypotrigona gribodoi* (Combey et al. 2013), and other unpublished results from our research group, including *Plebeia nigriceps* and *Mourella caerulea* in South Brazil and *Schwarziana quadripunctata*, in Southeast Brazil. All of these species are very different in various parameters related to their population dynamics, but gave similar results to those of the analysis we made of *Te. iridipennis* in India, except for the greater distances between populations than between species. The high discrimination rates in the cross-validation test and the comparison of the intra-group variability with the distances between the centroids of the groups also point in the same direction, indicating that these two populations form different phenotypic clusters and may belong to different taxonomical units.

Assam and Meghalaya are in a part of northeast India that is considered a hot spot of biodiversity. The area forms part of both Himalaya and Indo-Burma biodiversity hot spots, with unique and rich biodiversity (Mao et al. 2009). The area is characterized by grasslands, meadows, marshes and swamps, scrub forests, mixed deciduous forests, humid evergreen forests, temperate and alpine vegetation. This region also exhibits very rich diversity in orchids, zingibers, yams, rhododendrons, bamboos, canes, as well as wild relatives of cultivated plants, including citrus, banana, rice, sugarcane and pulses. It is home to about 50 % of India's biodiversity (Mao and Hynniewta 2000).

Other states, viz. Maharashtra, Karnataka, Kerala, Tamil Nadu and Andhra Pradesh, from where most of the stingless bee samples were collected in our study, form peninsular India. This region is characterized by large areas of cultivated land, with major crops such as cotton, oilseeds, cereals, sugarcane, tropical fruits and vegetables. The area also includes Western Ghats, which was recently recognized as a UNESCO heritage site, being one of the eight hottest hot spots of biodiversity in the world. It includes evergreen and semi-evergreen tropical rain forests, with several economically important tree species, including Indian rose wood (*Dalbergia sissoo* Roxb.), malbarkino (*Pterocarpus marsupium*), teak (*Tectona grandis* L.) and *Terminalia crenulata* Roth. In the elevated areas, coffee, tea, coconut, arecanut, pepper, rubber and cashew nut are widely cultivated.

These large contrasting environments probably fostered the development of a high level of variability. The effects of habitat variation and geographic distance on populations are difficult to separate and consequently determine if the current degree of differentiation is sufficient to indicate the existence of two species. Nonetheless, we strongly advise that the colonies in these two areas be managed as distinct entities because they constitute clear phenotypic clusters, independent of their status as one or two species. To test these hypotheses, it will be necessary to analyze more populations along a cline between them or implement an integrative approach, using other markers, such as DNA and ecological characters in an integrated approach, like the one used for delimiting species of the genus *Austroplebeia* (Halcroft et al. 2015). To reinforce the idea that these groups should be treated as two different units, we also cite the fact that nest architecture differs between colonies from Assam and Meghalaya and those from the other parts of the country. While the bees in Assam and Meghalaya construct the brood cells in layers, the bees in the other parts of the country construct the brood area in cluster format (supplementary Figures 3 and 4). The population of Karnataka also deserves some attention, since it was not statistically different from the population of Meghalaya, probably due to its wide intra-populational variability. More than one group may be in this sample and this subject will be treated in future research.

New taxonomic tools can be useful for discovery of species (Freitas et al. 2009). Nonetheless, a taxonomic review is needed for this group, including the construction

of an identification key and placement of these species within a taxonomic context. Novel morphological and molecular data would be very useful for this task, and this concept will probably guide the next steps of our research on this apparent species complex.

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