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Geometric morphometrics of the wing as a tool for assigning genetic lineages and geographic origin to *Melipona beecheii* (Hymenoptera: Meliponini)

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Abstract – The stingless bee *Melipona beecheii* presents great variability and is considered a complex of species. In order to better understand this species complex, we need to evaluate its diversity and develop methods that allow geographic traceability of the populations. Here we present a fast, efficient, and inexpensive means to accomplish this using geometric morphometrics of wings. We collected samples from Mexico, Guatemala, El Salvador, Nicaragua, and Costa Rica and we were able to correctly assign 87.1% of the colonies to their sampling sites and 92.4% to their haplotype. We propose that geometric morphometrics of the wing could be used as a first step analysis leaving the more expensive molecular analysis only to doubtful cases.

Melipona beecheii / geometric morphometrics / wing morphometrics / conservation / genetic lineages

1. INTRODUCTION

The importance of bees as pollinators in natural and agro-ecosystems has been well documented (Kremen et al. 2002; Klein et al. 2007). However, there is increasing evidence of declines of bee populations (Kremen and Ricketts

2000; Biesmeijer et al. 2006; Potts et al. 2010), and therefore there is a global concern for their conservation (Byrne and Fitzpatrick 2009; Brown and Paxton 2009; Freitas et al. 2009).

In Central America, especially in Mexico, *Melipona beecheii* is one of the most studied species of stingless bee due to its historical, economic, and ecological importance (May-Itzá et al. 2009). This species is distributed across the Mesoamerican tropics from Mexico to Costa Rica. Although it was widely used in the past for honey production, habitat deterioration and other threats have caused a steady decline in managed and feral populations, so that currently it is considered an endangered species (González-Acereto et al. 2006). Quezada-Euán et al. (2007)

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used morphometrics and microsatellite DNA to compare *M. beecheii* from Mexico and Costa Rica and detected substantial differences between these populations. These results were later confirmed and expanded to other populations based on variation in the ITS1 and ITS2 ribosomal and mitochondrial CO-1 genes among bees from Mexico, Guatemala, El Salvador, and Costa Rica (De la Rúa et al. 2007; May-Itzá et al., unpublished data). Thus, evidence from various studies has demonstrated considerable genetic differentiation between populations of *M. beecheii*, suggesting various genetic lineages and that *M. beecheii* could be in fact a complex of species (May-Itzá et al., unpublished data). This makes the establishment of conservation measures for such genetically different taxonomic units a priority (May-Itzá et al. 2009). It is also important to count on a rapid and economic method for the identification of specimens coming from the different regions. Till now, traditional morphometrics have been used to identify specimens of *M. beecheii* however, one problem with these methods is that the size component is an important part of the variation between samples and size can vary in stingless bees across time (Quezada-Euán et al. 2011).

Geometric morphometrics of wings has proven to be a powerful methodology for identifying specimens to genera and species in bees. In *Apis mellifera*, specimens have been accurately assigned to the subspecies level (Francoy et al. 2008; Tofilsky 2008; Francoy et al. 2009b), workers and males were correctly identified in stingless bees (Francoy et al. 2009a), cryptic species were detected in *Plebeia remota* (Francisco et al. 2008), and genetic variability among populations was also evaluated using this type of analysis (Mendes et al. 2007; Kandemir et al. 2009). Even though it is a simple technique for which only a stereomicroscope, a digital camera and a computer are needed, geometric morphometrics gives clear results that can be helpful as a first step towards answering questions that are normally resolved using molecular methods, which are much more expensive, require specialized personal and well-equipped laboratories. Additionally, important geometric morphometrics

can exclude the effects of size as it uses the relative positions of the plotted landmarks. Thus, environmental influences, such as parasitism and nutrition, can be eliminated or attenuated, concentrating on the variation at the genetic level (Monteiro and Reis 1999).

Here, we compared the patterns of wing venation of *M. beecheii* from Mexico, Nicaragua, Guatemala, El Salvador, and Costa Rica and correlated it to intra-specific genetic variability. The genetic lines that we used were already established for this species; our aim was to correlate geometric morphometric analyses of the wings molecular data and standard morphometrics analyses since geometric morphometric analyses has the potential to become a simple and reliable method for specimen identification.

2. MATERIAL AND METHODS

Samples of *M. beecheii* workers were collected from Mexico, Guatemala, Nicaragua, El Salvador, and Costa Rica in 2006 (Table 1). Each sample consisted of about 15 worker bees collected from the nest area from each colony. All samples were preserved in ethanol at -20°C awaiting further analysis.

The right forewings of five to ten bees per colony were mounted between microscope slides and photographed with a digital camera attached to a stereomicroscope.

We manually plotted ten homologous landmarks in the wing veins intersections (Figure 1) using the software tpsDig2, version 2.04 (Rohlf 2005). The images were Procrustes aligned (Bookstein 1991) and the mean configuration of the bees from a colony was used as a comparative parameter and the analysis was made at the colony level. A principal component analysis (PCA) was carried out using the relative Cartesian coordinates of each landmark after alignment. A forward stepwise analysis (tolerance 0.01; F to enter 1.00) using the same measures was carried out to determine discriminant functions, followed by a canonical analysis and then a cross-validation test to check the accuracy of the equations in identifying the colonies. We analyzed the groups in two different ways, labeling the colonies according to the geographic origin and also according to the ITS-1 haplotype of the colonies which was previously identified by May-Itzá et al. (2009). The

Table I. Collection sites, number of colonies of *M. beecheii* sampled, type of vegetation, and type of climate at each location.

Country	State	<i>N</i>	Type of vegetation	Type of climate	Geographic coordinates
México	Yucatán	10	Tropical sub-humid forest	Warm sub-humid	20°47'N 89°03'O
	Chiapas	4	Tropical humid forest	Warm moist	16°08'N 92°46'O
	Campeche	10	Tropical sub-humid forest	Warm sub-humid	19°50'N 90°31'O
Guatemala	Petén	2	Subtropical rainforest	Warm moist	16°54'N 90°17'O
	Retalhuleu	1	Subtropical moist forest	Warm moist	14°25'N 91°47'O
	Alta Verapaz	3	Subtropical rainforest	Temperate	15°35'N 90°08'O
	San Marcos	1	Warm subtropical rainforest	Warm sub-humid	14°55'N 91°54'O
	Chiquimula	2	Subtropical rainforest	Warm temperate dry	14°45'N 89°28'O
	Santa Rosa	1	Subtropical rainforest	Temperate	14°11'N 90°39'O
	Chalatenango	10	Subtropical sub-humid forest	Temperate	14°02'N 88°56'O
El Salvador, Nicaragua, Costa Rica	León	6	Subtropical dry forest	Tropical dry	12°30'N 86°39'O
	Heredia	1	Tropical rainforest	Fresh temperate dry	10°28'N 84°01'O
	Guanacaste	3	Tropical dry Forest	Tropical dry	10°29'N 85°21'O
	Alajuela	3	Tropical rainforest	Fresh temperate dry	10°23'N 84°26'O
	Puntarenas	2	Subtropical dry forest	Tropical dry	9°13'N 83°20'O
Total		59			

N number of colonies

statistical analyses were carried out using Statistica 6.0 (StatSoft 2001).

We also calculated the square Mahalanobis distances between the centroids of the groups and correlated it to the geographical distances between the populations using a Mantel test. A UPGMA dendrogram of morphological proximity was constructed using MEGA version 4.1 (Tamura et al. 2007) and the cophenetic correlation coefficient was calculated using NTSYS-PC version 1.5 (Rohlf 1989).

3. RESULTS

Six factors with eigenvalues greater than one were extracted in the PCA. Together, these six factors explained 75.77% of the data variability. The first two factors explained 43.61% of the variability, 28.67% by the first factor and 14.94% by the second factor. The colonies from Mexico

were placed mainly in the left-hand quadrant, while the other populations were placed mainly in the right-hand quadrant (Figure 2).

When analyzing the colonies that were labeled according to the ITS1 haplotype, the ones with ITS1-A haplotype (which are mainly from the Mexican regions) were also placed on the left-hand quadrant, with some colonies in the right-hand quadrant while the ones belonging to the ITS1-B haplotype were placed mainly in the right-hand quadrant. The one colony that presented the ITS1-C haplotype was placed in the lower right hand quadrant, at the edge of the distribution (Figure 2).

The first discriminant analysis, using the geographical origin of the colonies as labels, indicated that 13 Cartesian coordinates significantly contributed ($\alpha=0.05$) to separation of the groups according to geographical origin (Wilk's

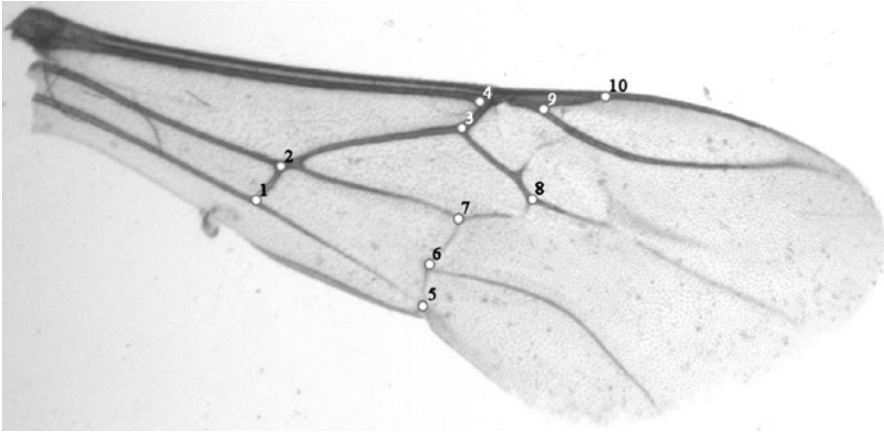


Figure 1. Forewing of *M. beecheii* worker with ten landmarks plotted in the vein junctions.

lambda=0.042 $P < 0.0001$). An analysis of Mahalanobis square distances between the centroids indicated that each group was significantly different from each other group ($\alpha = 0.05$) (Table II). The Mantel test indicated a strong correlation between the genetic and the geographical distances between the pairs of populations ($R = 0.864$ $P < 0.008$). The dendrogram of morphological proximity (Figure 3) placed the

Mexican population in an isolated branch, while the other four populations split in the other branch. This second branch was divided into two, one sub-branch with the populations from Costa Rica and Nicaragua and the other sub-branch, more distant, with the populations from Guatemala and El Salvador. The calculated cophenetic correlation coefficient was 0.74, indicating that the dendrogram is a good two-

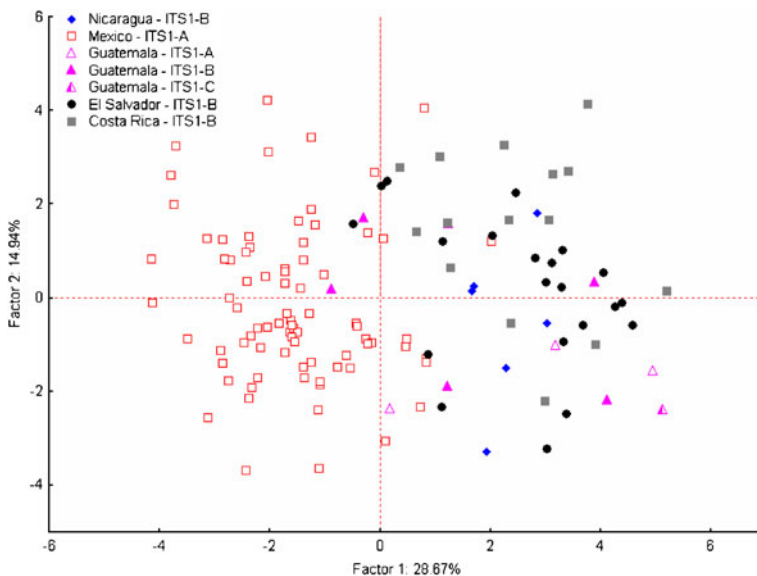


Figure 2. Principal component analysis of the five *M. beecheii* populations according to their geographic origin and ITS1 haplotype of the colonies.

Table II. Mahalanobis square distances between the centroids of the groups.

	Mexico	Guatemala	El Salvador	Costa Rica
Nicaragua	23.22	22.66	16.55	18.08
Mexico		18.57	19.96	36.01
Guatemala			8.33	21.49
El Salvador				15.64

All the groups were significantly different from each other ($P < 0.05$)

dimensional representation of the calculated distances (Rodrigues and Diniz-Filho 1998).

The discriminant functions and the aligned coordinates of the landmarks for each geographic population are listed in appendices I and II in the [supplementary online material](#). The discriminant analysis was able to correctly assign 87.1% of the colonies to their respective geographic group in the cross-validation test. The five groups were relatively well defined by geometric morphometrics (Figure 4). The 95% confidence ellipses show little superimposition among the groups (excluding the groups from Guatemala and El Salvador which are genetically similar to each other too; May-Itzá et al., unpublished data).

In the second discriminant analysis, in which we used the ITS-1 haplotype to discriminate the colonies, we excluded the sample that presented the ITS1-C haplotype because this kind of analysis is only suitable for groups with at least two colonies. This analysis indicated that nine Cartesian coordinates significantly contributed ($\alpha = 0.05$) to the separation of the two groups (Wilk's lambda = 0.256, $P < 0.0001$). The discriminant functions were able to correctly classify 92.4% of the colonies according to the respective

haplotype in the cross-validation tests. The mean configuration of the Cartesian coordinates of the colonies according to the ITS1 haplotype and the classification functions can be found in appendices III and IV in the [online supplementary material](#).

4. DISCUSSION

The analysis we conducted on the *M. beecheii* samples showed the efficiency of geometric morphometrics in correctly assigning the colonies to both geographic origin and their haplotype groups.

The PCA results (Figure 2) clearly indicated the formation of two separated clusters, one comprising the samples from Mexico and the other one with the samples from the other Central America countries. The formation of these clusters is in accordance with the results presented by May-Itzá et al. (2009) which demonstrated that the bees from Mexico present an ITS1 haplotype (ITS1-A) different from the bees from Central America (ITS1-B and ITS1-C). Indeed, if we look deeply in to the graph (Figure 2), we will verify that the group that

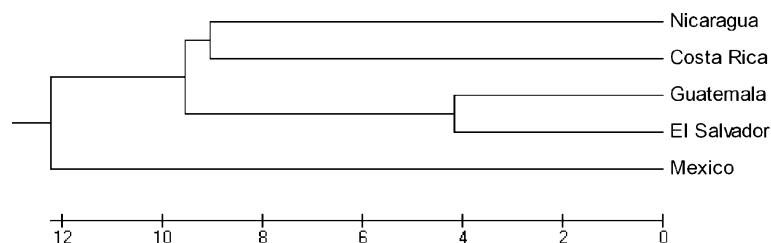


Figure 3. UPGMA dendrogram of morphological proximity constructed based on the Mahalanobis square distances between the centroids of the groups.

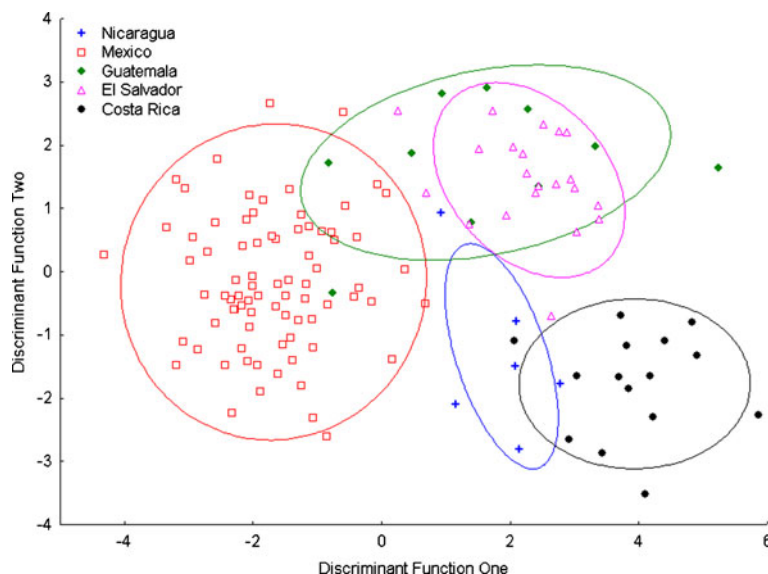


Figure 4. Discriminant analysis of the *M. beecheii* populations according to the geographical origin of the sampled populations.

presents the greater variability is the population of Guatemala. The points are widely spread in the right-hand quadrant and also in part of the left-hand quadrant. This variability is reflected in the ITS1 variability, since it is the only population that presents all the three haplotypes (May-Itzá et al. 2009). Since the bees collected in Guatemala are from different environmental areas, it may be a reflex of this variety where the bees were sampled.

The discriminant analysis (Figure 3) also indicated that there is also a discrimination of the groups according to the region where the bees were collected. When we compare the morphological distances between the groups, we can also see that they are strongly correlated with the geographical distances between the sampling sites. The short swarming ranges known for stingless bee species (Nogueira-Neto 1997) can help us to explain the local variation among the populations; this limited dispersal mechanism may contribute to the geographical isolation of the populations. The variability that we found indicates the existence of locally adapted ecotypes; it also corroborates conclusions made with various types of markers including standard

morphometrics and microsatellites (Quezada-Euán et al. 2007) RFLP and nuclear gene sequences, such as ITS2 (De la Rúa et al. 2007) and ITS1 (May-Itzá et al. 2009).

This variability may reflect the great variety of environments and selective pressures on this bee species along its wide range of distribution from North to Central America (Morrone 2006). A similar situation is found in *A. mellifera* populations along its original geographical distribution, which covers almost all of Africa and Europe and part of Asia (Ruttner 1988). This bee is classified in more than 24 subspecies and the existence of different evolutionary branches is confirmed by several markers, including morphometrics (Ruttner 1988), microsatellites (Estoup et al. 1995), mitochondrial DNA (Franck et al. 2000) and SNPs in the nuclear DNA (Whitfield et al. 2006).

The dendrogram of morphological proximity also supports the existence of different genetic lineages, with the population of Mexico in an isolated branch and the other populations subdivided in the other branch. The populations from El Salvador and Guatemala are placed very close to each other, supporting the conclusions based on

genetic data, which reveals that these populations are very similar (May-Itzá et al., unpublished data).

Another important point is the geographical traceability of the populations using the measures extracted from the wings. The correct assignment of almost 90% of the colonies to its original country is very promising for supporting measures for the conservation of these bees. Similar results of geographical traceability using geometric morphometrics of wings were also reported by Mendes et al. (2007) Francisco et al. (2008), and Bischoff et al. (2009).

Both the geometric morphometrics analyses and the studies made with other markers indicate that there are locally adapted ecotypes of *M. beecheii* that need protection in order to survive. The decline of pollinators (Winfree et al. 2009) may have catastrophic consequences for world food production. This decline, allied to the increasing demands for pollination with *A. mellifera* bees (Aizen et al. 2009; Aizen and Harder 2009) and the lack of knowledge about population dynamics and genetic structure of stingless bee populations make the study of native bee populations. In this context, the use of simple but informative tools like geometric morphometrics of wings can provide with a rapid and cheap method to evaluate inter and intra-specific variability across regions and allow to better focus conservation efforts. Here we present an alternative methodology to be used as a first step in the identification of these and other ecotypes instead of the more time consuming and expensive molecular methods, leaving this kind of analysis only to doubtful identifications.

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Morphométrie géométrique de l'aile : un outil pour attribuer des lignées génétiques et une origine géographique à *Melipona beecheii* (Hymenoptera: Meliponini).

Morphométrie / aile / conservation / méthode d'identification / lignée génétique / population

Zusammenfassung – Geometrische Morphometrie des Flügels als Werkzeug für die Zuordnung zu genetischen Linien und geographischem Ursprung bei *Melipona beecheii* (Hymenoptera: Meliponini)

Die stachellose Biene *Melipona beecheii* spielt in ökologischer, ökonomischer und historischer Hinsicht in ganz Mittelamerika eine bedeutsame Rolle. Die ausgedehnte geographische Verbreitung dieser Biene hat eine bemerkenswerte genetische Variabilität zur Folge, die mit molekularen Methoden gut untersucht werden kann. Heute wird davon ausgegangen, dass es sich bei *M. beecheii* in Wirklichkeit um einen Artenkomplex handelt, und es werden einige Anstrengungen unternommen, um diese bedrohte Gruppe zu erhalten. Zu diesem Zweck ist eine schnelle und preiswerte Methode zur Bestimmung von Proben aus verschiedenen Regionen des Verbreitungsgebiets wichtig. Wir stellen hier mit der geometrischen Morphometrie der Flügel eine effektive, schnelle und billige Alternative vor, mit der Völker von *M. beecheii* einer genetischen Linie zugeordnet werden können und darüber hinaus ihr geographischer Ursprungsort ermittelt werden kann. Wir haben dazu Proben im gesamten geographischen Verbreitungsgebiet der Art gesammelt und waren auf der Basis von Flügelmerkmalen in der Lage, 87.1% der Proben den Sammelorten sowie 92.4% dem korrekten genetischen Haplotyp zuzuordnen. Wir konnten auch bestätigen, dass ein Zusammenhang zwischen den morphologischen Distanzen der verschiedenen Gruppen und den geographischen Distanzen zwischen den Sammelorten besteht, was darauf hindeutet, dass zwischen den Populationen Isolation durch Distanz besteht. Auf der Grundlage dieser Ergebnisse schlagen wir vor, dass als ein erster Schritt in der Identifizierung unbekannter Proben die geometrische Morphometrie der Flügel durchgeführt werden sollte und damit wesentlich kostspieligeren molekularen Analysen auf zweifelhafte Fälle beschränkt bleiben können.

***Melipona beecheii* / geometrische Morphometrie/ Flügelmorphometrie / Erhaltung / genetische Linien**

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