Identification of stingless bees (Hymenoptera: Apidae) in Kenya using Morphometrics and DNA barcoding

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Summary

Stingless bees are important pollinators of wild plants and crops. The identity of stingless bee species in Africa has not been fully documented. The present study explored the utility of morphometrics and DNA barcoding for identification of African stingless bee populations and to further employ these tools to identify potential cryptic variation within species. Stingless bee population samples were collected from three ecological zones, namely Kakamega forest, Mwingi and Arabuko-Sokoke Forest, which are geographically distant and cover high, medium and low altitudes, respectively. Forewing and hind leg morphometric characters were measured to determine the extent of morphological variation between the populations. DNA barcodes were generated from the mitochondrial cytochrome *c*-oxidase I (*COI*) gene. Principal Component Analysis (PCA) on the morphometric measurements separated the bee samples into three clusters: 1) *Meliponula bocandei*, 2) *Meliponula lendliana* + *Plebeina hildebrandti*, 3) *Dactylurina schmidti* + *Meliponula ferruginea* black + *Meliponula ferruginea* reddish brown, but Canonical Variate Analysis (CVA) separated all the

species except the two morphospecies (*M. ferruginea* reddish brown and black). The analysis of the *COI* sequences showed that DNA barcoding can be used to identify all the species studied and revealed remarkable genetic distance (7.3%) between the two *M. ferruginea* morphs. This is the first genetic evidence that *M. ferruginea* black and *M. ferruginea* reddish brown are separate species.

Keywords: meliponines; cytochrome c oxidase I sequences; species identification; meliponiculture; East Africa

Short title: Identification of African Meliponinae

Introduction

Stingless bees play a vital ecological role as pollinators of many wild plant species and several crops (Heard, 1999; Heard & Dollin, 2000; Kakutani, Inoue, Tezuka, & Maeta, 1993; Kiatoko et al., 2014; Santos, Roselino, Hrncir, & Bego, 2009; Slaa et al., 2006). Stingless bee pollination is an important complement to honey bee pollination (Slaa et al., 2000). This is crucial as many parts of the world face honey bee colony losses caused by, among other factors, diseases and pests (Biesmeijer & Slaa, 2006; Muli et al., 2014; Neumann & Carreck, 2010; Pirk, Strauss, Yusuf, Demares, & Human, 2015). In addition, pollen and propolis from stingless bees have antibacterial, anti-inflammatory and free radical scavenging properties that can be utilized for medical purposes (Boorn et al., 2010; Libério, Pereira, & Araújo, 2009; Souza et al., 2006; Temaru & Shimura, 2007).

Similar to honey bees, stingless bees are vulnerable to habitat loss (through deforestation and habitat fragmentation), leading to reduced nest sites and food plants (Anguilet, 2015; Cortopassi-Laurino, 2006; Kajobe, 2007; Nkoba et al., 2012; Tornyie & Kwapong, 2015). In Kenya, stingless bees have been reported in Arabuko-Sokoke forest along the coast, Kakamega forest in western, and Mwingi woodlands in eastern Kenya. These three areas are exposed to deforestation due to demand for agricultural land and wood resources (Raina et al., 2011). To conserve these forests, the International Centre of Insect Physiology and Ecology (ICIPE) has established Meliponiculture projects for rural households living close to these forests. Species under domestication include *Meliponula ferruginea, Meliponula bocandei, Meliponula lendliana, Plebeina hildebrandti* and *Dactyrulina schmidti* (Eardley, 2004). Domestication has been envisaged for honey production and as an incentive for forest

conservation (Kiatoko, Raina, & Langevelde, 2016; Kiatoko et al., 2014; Macharia et al., 2010).

To further enhance domestication of stingless bees in Kenya, sound knowledge of their systematics is required. It is thus important to evaluate the genetic variation within the stingless bee populations to make sure the defined species are homogeneous taxonomic entities. This has implications on the success of colony propagation methods such as queen production, queen exchange, hive acceptance, and other important attributes of colony propagation (Cortopassi-Laurino, 2006; Slaa et al., 2006).

Stingless bees are usually identified using morphological features, nesting site, and nest architecture (Barbosa, Oliveira, Souza, & Carvalho, 2013; Batista, Ramalho & Soares, 2003; Nkoba et al., 2012). However, the use of morphological features is limited when some species or morphospecies closely resemble each other; for example, *M. ferruginea* reddish brown and *M. ferruginea* black, morphospecies which outwardly look similar in size and body structure (Eardley, 2004). However, authors have reported that the two morphospecies can be distinguished based on their nesting sites (Kajobe, 2007; Nkoba et al., 2012) and nest architecture (Nkoba, unpublished data). Nesting site alone as a means of identifying stingless bees is limited, as some species make use of the same nesting sites; for instance, *M. bocandei* and *M. ferruginea* nest in tree cavities, and *M. lendliana* and *P. hildebrandti* nest in underground cavities (Kajobe, 2007; Nkoba et al., 2012). The nest entrance architecture of *P. hildebrandti* and *M. ferruginea* black are similar (Nkoba, unpublished data). Detailed taxonomic analyses of African stingless bees are still lacking, for example the small differences between species and within species variations (Eardley & Kwapong, 2013; Michener, 2007; May-Itzá et al., 2012). Besides, interpretation of taxonomic keys requires skilled personnel and hence

the need to use robust tools such as morphometrics and molecular tools such as DNA barcodes.

Molecular sequence data have become more available to study taxonomy, population genetics, systematics and evolutionary trend in bees (Brito et al., 2013; Franck et al., 2004; Magnacca & Brown, 2010; Segura, 2000; May-Itzá et al., 2012). In addition, use of genetic distance evaluation and multivariate analysis of morphometric data have been applied to solve taxonomic problems in bees (Gibbs, 2009; Hurtado-Burillo et al., 2013; Sheffield et al., 2009). DNA barcoding has received much attention due to its ability to identify species (Hebert, Penton, Burns, Janzen, & Hallwachs, 2004; Hebert, Ratnasingham, & DeWaard, 2003; Hurtado-Burillo et al., 2013), including cryptic species (Hebert et al., 2004; Hurtado-Burillo et al., 2013). Morphometric tools combined with molecular tools (DNA barcoding) have been applied to identify complex species of the stingless bee *Melipona yucatanica* in Guatemala and Mexico (May-Itzá et al., 2010). In addition, morphometric measurements of the forewing (wing length and width, marginal and basal veins length) can be used in high-throughput protocol (Kaba et al., 2012), as is the case with DNA barcoding. High-throughput is an advantage over traditional identification methods that are slow and require a high level of expertise.

The main objective of this study was to explore the use of morphometrics and DNA barcoding as tools for the identification of stingless bee species that are the focus of domestication projects in different sites in Kenya. Our secondary objective was to identify some potential cryptic variation within species.

Materials and methods

Sampling sites

Stingless bee samples were collected from 2012 to 2014 in three ecological zones in Kenya, namely Kakamega, Mwingi and Arabuko-Sokoke, which are geographically distant and cover high, medium and low altitudes, respectively (Figure 1). These zones are areas where ICIPE has established various projects on stingless beekeeping.

Kakamega forest is an indigenous forest in western Kenya (0°09'N, 34°50'E), supporting high biodiversity (Zimmerman, 1972) including bees (Gikungu, 2006; Nkoba et al., 2012). It lies between 1500 - 1600 m above sea level (Tsingalia & Kassily, 2009), with an average annual rainfall of 1200 mm-1700 mm. Kakamega forest is the easternmost remnant of the rainforest found in the Democratic Republic of Congo and parts of West Africa. The surrounding human population density exceeds 300 persons per km², which is considered a high density (Tsingalia & Kassily, 2009).

Mwingi is found in the Eastern region of Kenya (0°51′S, 38°22′E) and lies between 600 - 900 m above sea level. The locality covers an area of 10,030.30 km^2 (Njoroge, Kaibui, Njenga, & Odhiambo, 2010) and is a semi-arid zone with an average annual rainfall range of 400 – 800 mm and temperatures that vary throughout the year between 14-34ºC (Njoroge et al., 2010; Opiyo et al., 2011). Large areas are occupied by grasslands and shrubs (Kaloi, Tayebwa, & Bashaasha, 2005).

Arabuko- Sokoke forest (3°20′ S, 39°50′ E) is a dry coastal forest and is home to endangered biodiversity (Glenday, 2008). It is a protected forest and a key biodiversity hotspot (Muriithi & Kenyon, 2002; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). The forest occupies an area of 420 km^2 and lies between 0 - 135 m above sea level. It has a bimodal rainfall pattern with average annual rainfall ranging between

600 – 1000 mm. The communities living around the forest are mainly subsistence farmers who rely on the forest for timber and firewood.

Sample processing and morphometric measurements

Four hundred and ninety seven (497) stingless bees from five species were collected for morphometric analysis (Table 1). *Meliponula ferruginea* black was sampled from Kakamega (140 specimens) and Mwingi (12 specimen). *Meliponula ferruginea* reddish brown was sampled from Kakamega (177 specimens) and Arabuko sokoke (30 specimens). *Dactylurina schmidti* was sampled from Arabuko-sokoke (24 specimens) and is only found in this coastal region. *Meliponula lendliana* (66 specimens), *M. bocandei* (36 specimens) and *P. hildebrandti* (12 specimens) were all sampled from Kakamega (Table 1). All specimens were identified to species level at the National Museum of Kenya using the key developed by Eardley (2004).

Each stingless bee specimen was dissected under the microscope to remove the right forewing and right hind leg. The legs and wings were mounted on 2 mm slides in Canada balsam and dried in the oven at 37^{o} C for 3 weeks using the protocol described by Billah et al. (2008). Images of mounted specimens were taken using Leica EZ4D stereomicroscope (Leica Microsystems Limited, Switzerland) under magnification of 20X (Figure 2 a–c).

A description of observable features of the main forewing and hindwing veins, and hind legs was recorded (Table 2). Measurements were taken using the microscope accompanying software LAS EZ, version 1.4.0. Measurements included forewing length (WL), forewing width (WW), distances between some selected forewing veins, V3–V11, and leg morphometrics; tibia length (TL), tibia width (TW), and femur length (FL) (Figure 3A and B). Each measurement was taken in triplicate (to an accuracy of

0.001 mm). Voucher specimens are preserved at the African Reference Laboratory for Bee Health at ICIPE in Nairobi, Kenya.

DNA extraction, amplification of the barcoding region and sequencing

Genomic DNA was extracted from individual stingless bees' legs using the DNeasy Blood and Tissue kit (Qiagen, GmbH-Hilden, Germany) by means of tissue extraction protocol, and the final elution volume was 80 µl. The extracted DNA was stored at –20 °C until required for amplification. The universal primer pair LCO1490 and HCO2198 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994) were subsequently used to amplify a 650 bp fragment of the *COI* gene. PCR was carried out in a total reaction volume of 25 µl containing 0.5 pmol of each primer, 10 mM Tris-Cl, pH 8.3 and 50 mM KCl, 1.5 mM MgCl2, 2.5 mM dNTPs, 2 µl of the DNA template and 1 unit of *Taq* DNA polymerase (Genscript Corp, Piscataway, NJ). PCR standard cycling conditions of 3 min at 94 °C, then 35 cycles of 30s at 94 °C, 30s at 47 °C and 30s at 72 °C, followed by a final elongation step of 10 min at 72 °C were used. The PCR products were visualized on a 1% agarose gel stained using ethidium bromide. The products were purified using QIAquick PCR purification kit (Qiagen, GmbH-Hilden, Germany) according to the manufacturer's instructions and subsequently sequenced bi-directionally using ABI 3700 genetic analyzer. The *COI* sequences were submitted to the Barcode of Life database (BOLD) and deposited in GenBank (Table 1).

Multivariate analyses of morphometrics

Morphometric analyses were performed using R version 3.2.1 (R Development Core Team, 2015). Principal Component Analysis (PCA), a multivariate method that assumes that all samples are from a single population was used to analyse the morphometric measurements to identify group clusters. Data were log transformed

(log_{10}) before analysis to stabilize the variance and normalize the variables (Keene, 1995). The first and second Eigen values were considered in the interpretation of the PCA output, as they were associated with much of the variation (>70%) in the measured variables. Character loadings were obtained for the first two principal components, to provide insight on the correlation of each character with the principal component. The first two principal component scores were plotted for the PCA on wing measurements only and on wing and leg measurements. Since PCA may not reveal all groups even if they exist, the log-transformed data were also subjected to Canonical Variate Analysis (CVA) to analyse the group structure as known a priori (using 'species' as a prior for each specimen). In addition, Mahalanobis squared distances (D^2) between species were computed. D^2 is a measure of divergence or distance between a pair of groups within the multivariate character space, in the presence of correlation among variables (Mahalanobis, 1936). D^2 was calculated to complement PCA and CVA plots, and the genetic distance table.

Phylogenetic analysis

Ninety-five *COI* sequences (Table 1) were assembled and edited using BioEdit version 7.25 (Hall, 1999). A maximum likelihood phylogenetic tree was produced in MEGA6 (Tamura et al., 2013) using the Tamura 3-parameter substitution model (Tamura, 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+ *G*, parameter = 0.5119)). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All

positions containing gaps, and missing data, were eliminated. There were a total of 646 positions in the final dataset. Two *Apis melifera scutellata COI* sequences were obtained from BOLD (sequence ID: KINS944-10.COI-5P and KINS940-10.COI-5P) and used as outgroup to root the phylogenetic tree. In addition, DNA barcode gap analysis was carried out within BOLD systems analysis tools to delineate the species (Puillandre et al., 2012). It is a useful tool to detect cryptic variation between taxa that are morphologically identical.

Results

Observable features of hindwings, forewings and hind legs

Examination of the specimens' forewings, hindwings, and hind legs uncovered new morphological characters distinguishing the species. The second submarginal cell is partially closed in *P. hildebrandti*, but open in all other species. The hind leg of *M. ferruginea* reddish brown is reddish brown while it is black in *M. ferruginea* black (Figure 2). We observed a unique characteristic in *M. lendliana* where vein Rs extends to the wing margin (Figure 1S). In addition, *P. hildebrandti* has seven hamuli and occasionally six in some samples, while *M. lendliana* has six (Figure 2S). In the hindwings of *P. hildebrandti*, the basal vein is sharply curved whereas it is gently curved in *M. lendliana* (Table 2). The 2nd abscissa of vein Rs in the *M. ferruginea* reddish brown forewing is incomplete and faint whilst in *M. ferruginea* black, it is distinct and complete. *Meliponula bocandei* had the longest wing, which is in agreement with findings of Eardley (2004). *Meliponula bocandei* had the highest number of hamuli (nine) in the hind wing.

Morphometrics

Principal Component Analysis showed that the first two principal components explained 82.3% of the total variation in the wing morphometric measurements (PC1 = 65.7% and PC2 = 16.6%) (Table 1S). Projection of the data on the first two principal components revealed three groups namely: 1) *M. bocandei*; 2) *M. ferruginea* black, *M. ferruginea* reddish brown and *D. schmidti*; and 3) *P. hildebrandti* and *M. lendliana* showing partial separation (Figure 4a and c). However, projection of the data on the first two canonical variates (CV) showed that each species were a distinct group except for *M. ferruginea* black, *M. ferruginea* reddish brown (Figure 4 b, d). The first two canonical variates contributed a total of 93.6% (CV1 = 68.4% and CV2 = 25.2%). The standardized canonical coefficients (Table 2S) were higher for wing length, radius and cubitus on CV1 and for radius, basal veins, wing length and vannal vein on CV2. PCA and CVA plots did not differ for wing measurements alone and wing and leg measurements considered together (Figure 4 and Figure 3S respectively).

The largest Mahalanobis squared distance (D^2) was between *M. bocandei* and *P. hildebrandti* (D^2 = 284.03) while the smallest distance was between *M. ferruginea* reddish brown and black (D^2 = 2.2) (Table 3).

DNA Barcoding (Intra and Inter-specific genetic distance and Barcode gap analysis)

A total of 95 sequences were generated from 95 stingless bee samples, each of which consisted of 640 base pairs. Various mitotypes were identified for the five species (considering the two *M. ferruginea* morphs separately) as follows: *M. ferruginea* reddish brown, 7 different mitotypes amongst 36 sequences generated (6 in Kakamega and 1 in Arabuko-Sokoke); *M. ferruginea* black, 2 different mitotypes (1 in

Mwingi and 1 in Kakamega) amongst 21 sequences generated; *M. lendliana*, 3 different mitotypes amongst 24 sequences generated; *D. schmidti*: 1 mitotype amongst 4 sequences generated; *P hildebrandti*, 1 mitotype amongst 4 sequences generated; and *M. bocandei*, 1 mitotype amongst 6 sequences generated. A clade was considered well supported if bootstrap percentage was greater or equal to 50% (Baldauf, 2003). *Meliponula species* separated forming two clades: *M. ferruginea* and *M. lendliana*. Within *M. ferruginea*, the two morphs (reddish brown and black) separated into two clades with *M. ferruginea* black collected from Mwingi and Arabuko-Sokoke in one clade and *Meliponula ferruginea* reddish brown collected from Arabuko-Sokoke and Kakamega in the other (Figure 5). This distinction was also observed for *M. ferruginea* reddish brown and black Kakamega populations. The genetic distance between *M. ferruginea* reddish brown and black from Kakamega (7.3%) was greater than the distance between *M. ferruginea* black from Kakamega and Mwingi (1.4 %) (Table 4). Mean distances within groups ranged from 0 - 0.04% and between groups ranged from 7 - 17%. Barcode gap analysis estimated a mean intraspecific genetic distance (genetic distance between individuals belonging to the same species) of 0.31 (minimum - maximum range: 0 - 1.6), whereas the estimated mean distance to the Nearest Neighbor was 8.97 (minimum- maximum range: 4.93 - 17.01).

Discussion

This study aimed at combining morphological features, morphometrics and molecular methods in the identification of stingless bees. We found that though *M. lendliana* and *P. hildebrandti* are superficially similar in size (Eardley, 2004), *M. lendliana* shows a unique characteristic where vein Rs extends to the margins of the

forewing. There was remarkable difference in the 2nd abscissa of Rs vein in the forewing, which is faint in *M. ferruginea* reddish brown but distinct and complete in *M. ferruginea* black. In previous studies *M. ferruginea* reddish brown and *M. ferruginea* black have been named as "morphospecies" due to different colour of the metasoma which is brown and black, respectively (Eardley, 2004; Kajobe, 2007). Until now, few morphological descriptions have been carried out (Michener, 2007; Eardley, 2004). The revision by Eardley (2004) provides a description and identification key, showing the distribution maps, forewings, heads and genitalia figures for the worker bees of African stingless bees.

Morphometric analysis has been tested and successfully used to study honey bees and Meliponini populations in different parts of the world (Gibbs & Dumesh, 2013; Packer et al., 2009). Our results showed that some species that could not separate on PCA analysis separated well in CVA. For instance, as observed from the PCA plots *P. hildebrandti* and *M. lendliana* could not separate as they are similar in size and thus only partially separate, however the two separated on CVA plots. In addition, *D. schmidti* and *M. ferruginea* did not separate on PCA analysis. The same data subjected to CVA led to separation of *D. schmidti* from *M. ferruginea*.

One way of identifying stingless bees is by use of nest entrance, nest architecture, and nesting site (Roubik, 2006; Raina et al., 2011). For example, though *D. schmidti* and the two *M. ferruginea* morphs have very similar morphometry, their nesting behavior is distinct. *Dactylurina schmidti* constructs external nest on tree branches while *M. ferruginea* reddish brown nests in mud walls and trees, and *M. ferruginea* black nests in trees (Eardley, 2004; Nkoba et al., 2012). *Meliponula ferruginea* reddish

brown has also been observed to nest in deserted termite mounds and in the ground (Tornyie & Kwapong, 2015).

DNA barcoding showed clear separation between *M. ferruginea* reddish brown and *M. ferruginea* black into two separate clades. The genetic distance between *M. ferruginea* black collected from Kakamega and *M. ferruginea* black from Mwingi was low (1.4%), within expected species variation for barcode data based on *COI* for most animals (Hebert et al., 2003) and in accordance with the Barcode gap estimate for within species genetic variation (<1.6%). The genetic distance between *M. ferruginea* black and *M. ferruginea* reddish brown collected from Kakamega, in sympatry, was 7.3%. On the other hand the genetic distance between *M. ferruginea* reddish brown from Kakamega and *M. ferruginea* black from Mwingi was 5.6%. These results suggest that the two morphs are genetically distinct. Moreover, DNA barcoding of bees of the world classified such genetic distances (>4.93%) as interspecific (Packer et al., 2008). This strongly supports the hypothesis that *M. ferruginea* reddish brown and *M. ferruginea* black are distinct species. Based on nest architecture and nesting habits recorded in Uganda, Kajobe (2007) showed that the nest shape for *M. ferruginea* black is circular tube while that of *M. ferruginea* reddish brown is circular and tapers; they both nested in specific tree species. However, results of a recent study carried out in Kenya, reported that *M. ferruginea* black nests in indigenous trees in the forest while *M. ferruginea* reddish brown nests in mud walls in homesteads as well as indigenous trees (Nkoba et al., 2012). In addition, *M. ferruginea* reddish brown showed nest aggregation while *M. ferruginea* black nests were dispersed (Kajobe, 2007; Nkoba et al., 2012). Cerumen used in construction of entrances of *M. ferruginea* reddish brown nest is

reddish brown while the one used in *M. ferruginea* black is dark brown (Nkoba et al., 2012).

Our results show low intraspecific variation compared to interspecific variation between the five species (7–17%), thus DNA barcoding can be used to identify the stingless bee species. In addition, DNA barcoding separated the species according to their locations, for example, *M. ferruginea* black collected from Kakamega and Mwingi separated on the phylogenetic tree in different branches similar to *M. ferruginea* reddish brown collected from Kakamega and Arabuko-sokoke. This is in accordance to previous reports which shows that DNA barcoding gap can be applied to separate species over geographical and morphological scales (Čandek & Kuntner, 2014). A major advantage of DNA barcoding is that it is amenable to high throughput identification, with minimal expertise in taxonomy. The *COI* sequence analysis revealed cryptic genetic variation within the species *M. ferruginea* reddish brown and black. Several authors have suggested recently that *M. ferruginea* reddish brown and black would be two separate species (based on colour and nest architecture) (Kajobe, 2007) while Eardley (2004) regarded the two as synonymous. The substantial genetic variation between *M. ferruginea* reddish brown and *M. ferruginea* black as well as their colour and nest architecture differences strongly suggests they are separate species. This result is the first genetic evidence that *M. ferruginea* reddish brown and *M. ferruginea* black are two distinct species.

Our results demonstrate that DNA barcoding can be used for the identification of *M. bocandei, M. lendliana, M. ferruginea, D. schmidti* and *Plebeina hildebrandti*. Moreover the CVA analysis of the morphometric variation measured revealed that our morphometric measurements are in complete accordance with the classical

identification procedure based on morphological features. However, without prior knowledge about the species identity, our morphometrics analysis (PCA) could not distinguish *D. schmidti* from *M. ferruginea*. Thus, morphometrics alone cannot be used to identify all of the stingless bees studied here when the species identity is completely unknown. Species identification using DNA barcoding approach would thus be a more practical tool than morphometrics for individuals lacking the expertise in morphological identification of stingless bees.

We conclude that morphometrics could not distinguish *M. ferruginea* reddish brown and *M. ferruginea* black, while DNA barcoding revealed cryptic genetic variation within the two. DNA barcoding is therefore an important tool for identification of African stingless bee populations. In addition wing morphometrics alone (without leg morphometrics) are adequate for studying morphometric variation in African stingless bees. Further, we recommend that *M. ferruginea* reddish brown and black need to be studied further using other molecular markers.

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Supplementary materials

Table 1S. Eigen values and coefficients (loadings) of the first two principal components (PC1 and PC2) for the log-transformed wing measurements data for the five stingless bees; *M. bocandei*, *M. lendliana*, *P. hildebrandti*, *D. schmidti* and *M. ferruginea* reddish brown and black.

Table 2S. Standardized canonical coefficient for canonical variate analysis on log-transformed wing measurement data for five stingless bee; *M. bocandei*, *M. lendliana*, *P. hildebrandti*, *D. schmidti* and *M. ferruginea* reddish brown and black.

Figure 1S. The distal end of vein Rs as viewed on the forewing. a: Forewing showing the position of distal end of vein RS. b: *M. bocandei*. c: *M. ferruginea* black. d: *M. ferruginea* reddish brown. e: *M. lendliana*. f: *P. hildebrandti*. g: *D. schmidti*. The vein Rs is extended in *M. lendliana* (e).

Figure 2S. Number of hamuli on the hind wing of the five species. a: Hind wing showing the position of Hamuli. b: *M. bocandei*. c: *M. ferruginea* black. d: *M. ferruginea* reddish brown. e: *M. lendliana*. f: *P. hildebrandti*. g: *D. schmidti*. *Plebeina hildebrandti* has seven/ six hamuli.

Figure 3S (e-h). Principal components plots (e & g) and Canonical Variate plots (f & g) of the wing + legs morphometric measurements. PCA plot separated the species into three major groups. 1. *M. bocandei* stands alone. 2. *D. schmidti*, *M. ferruginea* reddish brown and black did not separate. 3. Partial separation of *M. lendliana* and *P. hildebrandti*. CVA plot separated all species except *M. ferruginea* black and reddish brown

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15 **Table 1.** Sampling sites, number of specimens collected from each site and their respective BOLD and Genbank Accession numbers.

Site	Species	Number of specimens		BOLD Accession Numbers	Genbank Accession Numbers
		Morphometrics	*COI* sequences		
Kakamega	***M. ferruginea*** black	140	16	KINS3989-13, KINS3990-13,KINS3991-13, KINS3993-13 BOFAS093-15, BOFAS095-15, BOFAS097-15, BOFAS100-15, BOFAS101-15, BOFAS164-15, BOFAS166-15, BOFAS184-15, BOFAS187-15, BOFAS188-15, BOFAS207-15, BOFAS208-15	KU146625, KU146619,KU146629, KU146617, KU146635, KU146628,KU146620, KU146632, KU146662, KU146630,KU146624, KU146621 KU146622, KU146618,KU146692 ,KU146623
	M. lendliana	66	24	KINS4004-13, KINS4005-13, KINS4006-13, KINS4007-13 KINS4008-13,KINS4009-13,BOFAS138-15,BOFAS139-15 BOFAS141-15,BOFAS143-15,BOFAS144-15,BOFAS146-15,BOFAS150-15,BOFAS153-15, BOFAS154-15,BOFAS155-15, BOFAS156-15,BOFAS157-15, BOFAS158-15, BOFAS159-15, BOFAS160-15,BOFAS162-15,BOFAS201-15,BOFAS202-15	KU146682,KU146680,KU146685,KU146673 KU146674,KU146681,KU146684,KU146690 KU146611,KU146686,KU146689,KU146608 KU146679,KU146687,KU146672,KU146683 KU146688,KU146691,KU146675,KU146678 KU146677,KU146676,KU146694,KU146693
	M. ferruginea reddish brown	177	31	BOFAS051-08, BOFAS052-08,BOFAS053-08, BOFAS054-08,BOFAS055-08, BOFAS076-08, BOFAS103-15, BOFAS109-15,BOFAS110-15, BOFAS111-15,BOFAS112-15, BOFAS114-15,BOFAS122-15, BOFAS123-15,BOFAS124-15, BOFAS127-15,BOFAS128-15, BOFAS130-15,BOFAS131-15, BOFAS132-15,BOFAS170-15, BOFAS171-15,BOFAS172-15, BOFAS176-15, BOFAS179-15, BOFAS180-15,BOFAS181-15, BOFAS197-15,BOFAS198-15, BOFAS210-15,BOFAS211-15,	KU146646,KU146667,KU146666,KU146648 KU146665, KU146664, KU146653,KU146652, KU146658, KU146641,KU146643, KU146651,KU146649, KU146670,KU146657, KU146668,KU146647, KU146615,KU146616, KU146640,KU146656, KU146661,KU146663, KU146655,KU146659, KU146671,KU146669, KU146645,KU146638, KU146644,KU146639
	M. bocandei	36	6	BOFAS140-15, BOFAS145-15,BOFAS147-15, BOFAS148-15,BOFAS149-15, BOFAS182-15	KU146607, KU146612,KU146609, KU146613,KU146610, KU146614
	P. hildebrandti	12	4	KINS3986-13, KINS3987-13,KINS3988-13, BOFAS116-15	KU146698, KU146696,KU146697,,U146695
Arabuko-Sokoke	***D. schmidti***	24	4	BOFAS036-08, OFAS037-08,BOFAS038-08, BOFAS039-08	KU146579,KU146577,KU146578,KU146580
	M. ferruginea reddish brown	30	5	BOFAS077-08, BOFAS078-08,BOFAS079-08, BOFAS080-08,BOFAS102-15	KU146637, KU146642,KU146636, KU146650, KU146654
Mwingi	***M. ferruginea*** black	12	5	BOFAS058-08, BOFAS059-08 BOFAS060-08, BOFAS056-08 BOFAS057-08	KU146627, KU146634 KU146631, KU146626 KU146633

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Table 2. Observable features of the hind legs, forewing and hind wing of stingless bee species under study; *M. bocandei*, *M. lendliana*, *M. ferruginea*, *D. schmidti* and *P. hildebrandti*

Morphological features		Stingless Bee Species					
		M. bocandei	***M. ferruginea*** black	***M. ferruginea*** reddish brown	***M. lendliana***	***P. hildebrandti***	***D. schmidti***
Fore wing	**Colour**	Dark brown	Dark brown	Dark brown	Dark brown	Dark brown	More Dark brown
	Veins, C, Rs, M, R, M+Cu, Cu-v, V, Rs+M, Cu	+	+	+	+	+	+
	2nd Abscissa of Rs	Distinct and Complete	complete	incomplete	incomplete	faint	faint
	2nd submarginal cell	open	open	open	open	Closed partially	open
	1r-m	faint	-	-	-	faint	-
	Rs	Not extended	Not extended	Not extended	Extends to wing margin	Not extended	Not extended
	1st and second Cubital cell	+	+	+	+	+	+
Hind wing	**Rs**	+	+	+	faint	+	+
	Number of Hamuli	9	6	6	6	6/7	6
	Cubital cell	complete	complete	complete	Not well defined	complete	complete
	Radial cell	+	+	+	Not very clear	+	+
	Jugal lobe	+	+	+	+	+	+
	Vannal lobe	+	+	+	+	+	+
	Vein V	+	+	+	+	+	+
	Basal vein	+	+	+	+ (gently curved)	+ (sharply curved)	+
Hind	**Basitarsus**	Orange yellow	black	reddish brown	Orange yellow	Dark brown	Dark brown

leg	**Femur**	Very hairy	hairy	hairy	slightly hairy	hairy	hairy

The vein terminology used is the Comstock–Needham terms as used by Michener, 2007, the terms used in colour description adapted from Eardley, 2004: + = present; - = absent

R = Radius; RS = radial sector; M = basal vein; M + Cu = medial–cubital vein; Cu = cubitus;

Table 3. Mahalanobis Squared distances (D^2) based on wings morphological characters between clusters representing the five stingless bee species.

Species	***M. bocandei***	***M. lendliana***	***P. hildebrandti***	***D. schmidti***	***M. ferruginea*** black
M. lendliana	245.4	-			
P. hildebrandti	284.03	51.5	-		
D. schmidti	92.1	69.9	95.2	-	
M. ferruginea black	94.3	64.4	131.3	26.4	-
M. ferruginea reddish brown	76.9	68.6	133.4	22.4	2.2

26 **Table 4.** Estimates of genetic distance based on *COI* sequences between groups generated by Mega 6 program (Tamura et al., 2013).

Species	***Apis m. scutellata***	***D. schmidti***	***M. bocandei***	***M. ferruginea* black, Mwingi**	***M. ferruginea* black, Kakamega**	***M. ferruginea* reddish brown , Kakamega**	***M. ferruginea* reddish brown , Arabuko-Sokoke**	***M. lendliana***
D. schmidti	0.218	-						
M. bocandei	0.261	0.114	-					
***M. ferruginea* black, Mwingi**	0.278	0.110	-0.152	-				
***M. ferruginea* black, Kakamega**	0.279	0.118	0.159	**0.014**	-			
***M. ferruginea* reddish brown , Kakamega**	0.262	0.116	0.159	0.056	**0.073**	-		
***M. ferruginea* reddish brown , Arabuko-Sokoke**	0.273	0.115	0.168	0.063	0.071	0.054	-	
M. lendliana	0.283	0.116	0.156	0.141	0.147	0.155	0.137	-
P. hildebrandti	0.230	0.085	0.121	0.130	0.136	0.126	0.137	0.128

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Figures

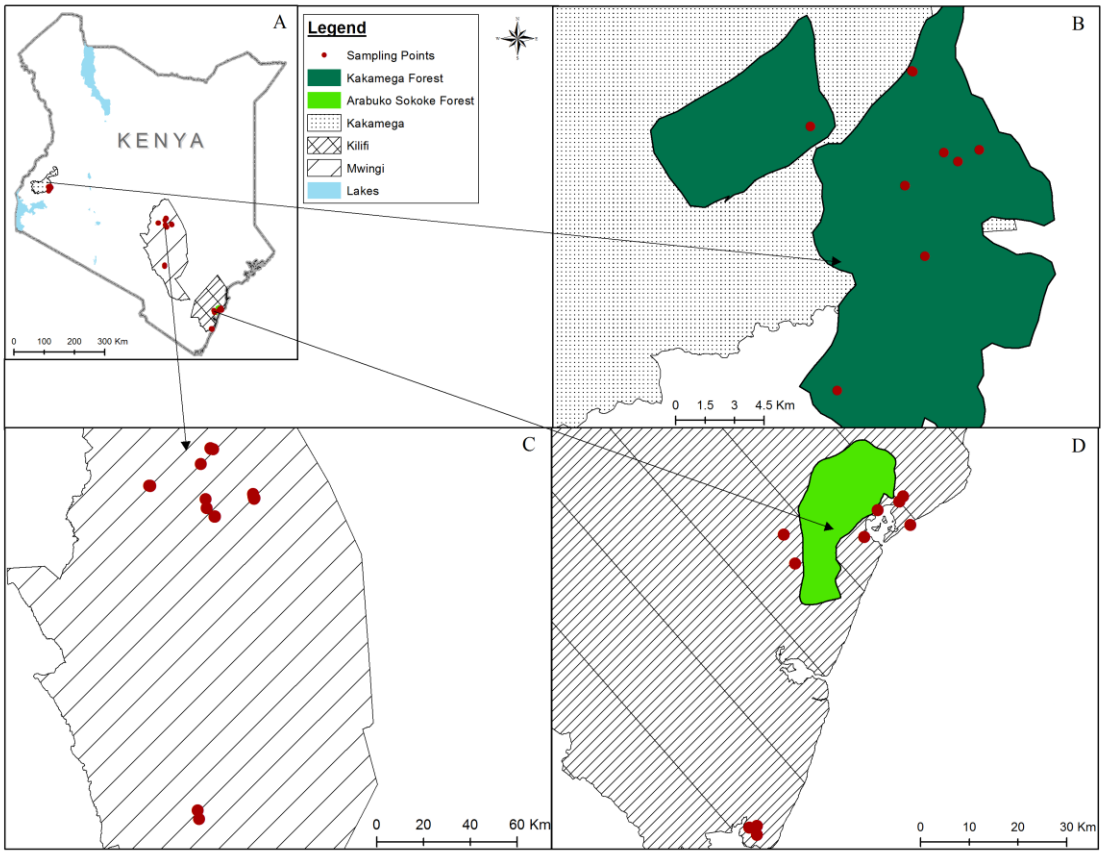

Figure 1. Localisation of sampling sites. A: general map of Kenya showing the three sampling areas (Kakamega forest, Mwingi and Arabuko-Sokoke forest). B: sampling sites in Kakamega forest. C: sampling sites in Mwingi. D: sampling sites in Arabuko-Sokoke forest.

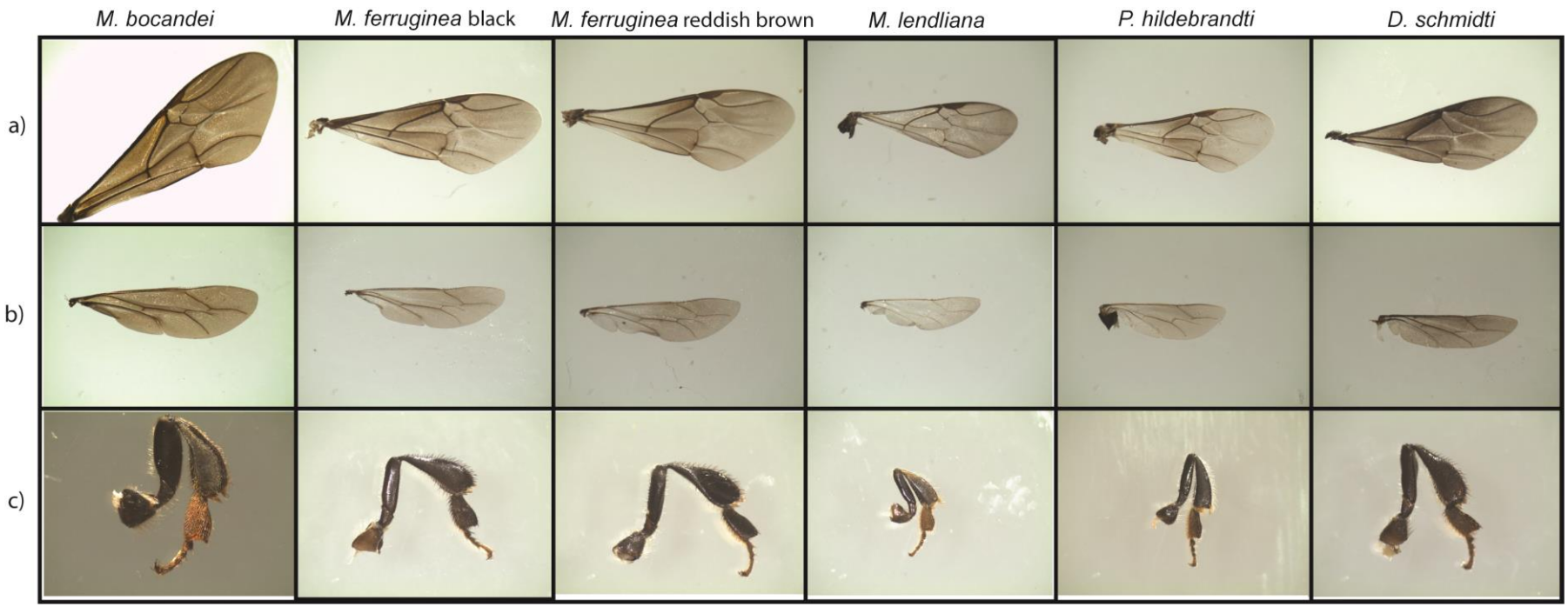

Figure 2. **Photos of the Fore wing, Hind wing and hind leg of the five species showing the similarities and variation between species.** a = Forewing b = Hindwing c = Hind leg. *M. bocandei* has the longest wing. *M. ferruginea* black and reddish brown and *D. schmidti* have similar fore wings and hind leg. *Plebeina hildebrandti* and *M. lendliana* have wings almost the same size.

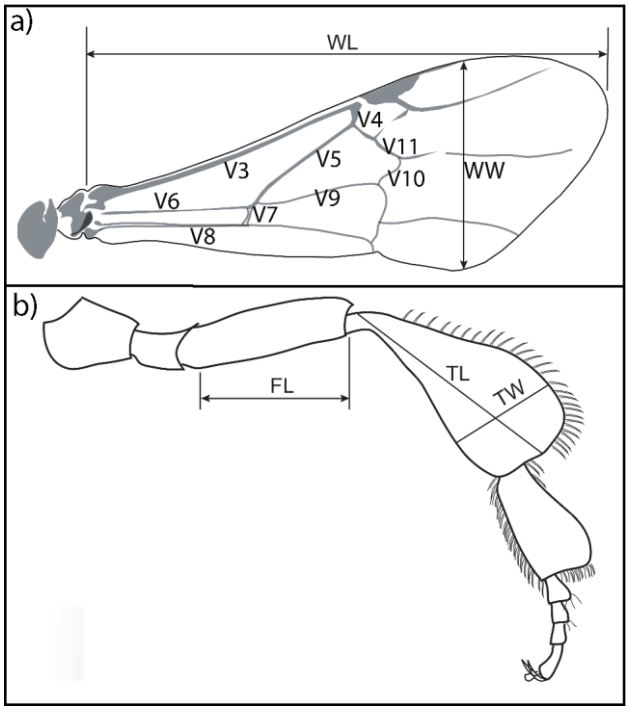

Figure 3. Schematic representation of the right forewing and the right hind leg presenting morphometric characters of interest. (A) Right forewing showing veins used in morphometrics studies. WL = wing length; WW = wing width; V3 = Marginal vein (R); V4 = radial sector (RS); V5 = basal vein (M); V6 = medial–cubital vein (M + Cu); V7 = cubitus (Cu); V8 = V; V9 = Cu; V10 = 1m - cu; V11 = Rs + M. (B) Right hind leg of a stingless bee**.** FL = femur length; TL = tibial length; TW = tibial width. *The terms are adopted from (Michener, 2007)

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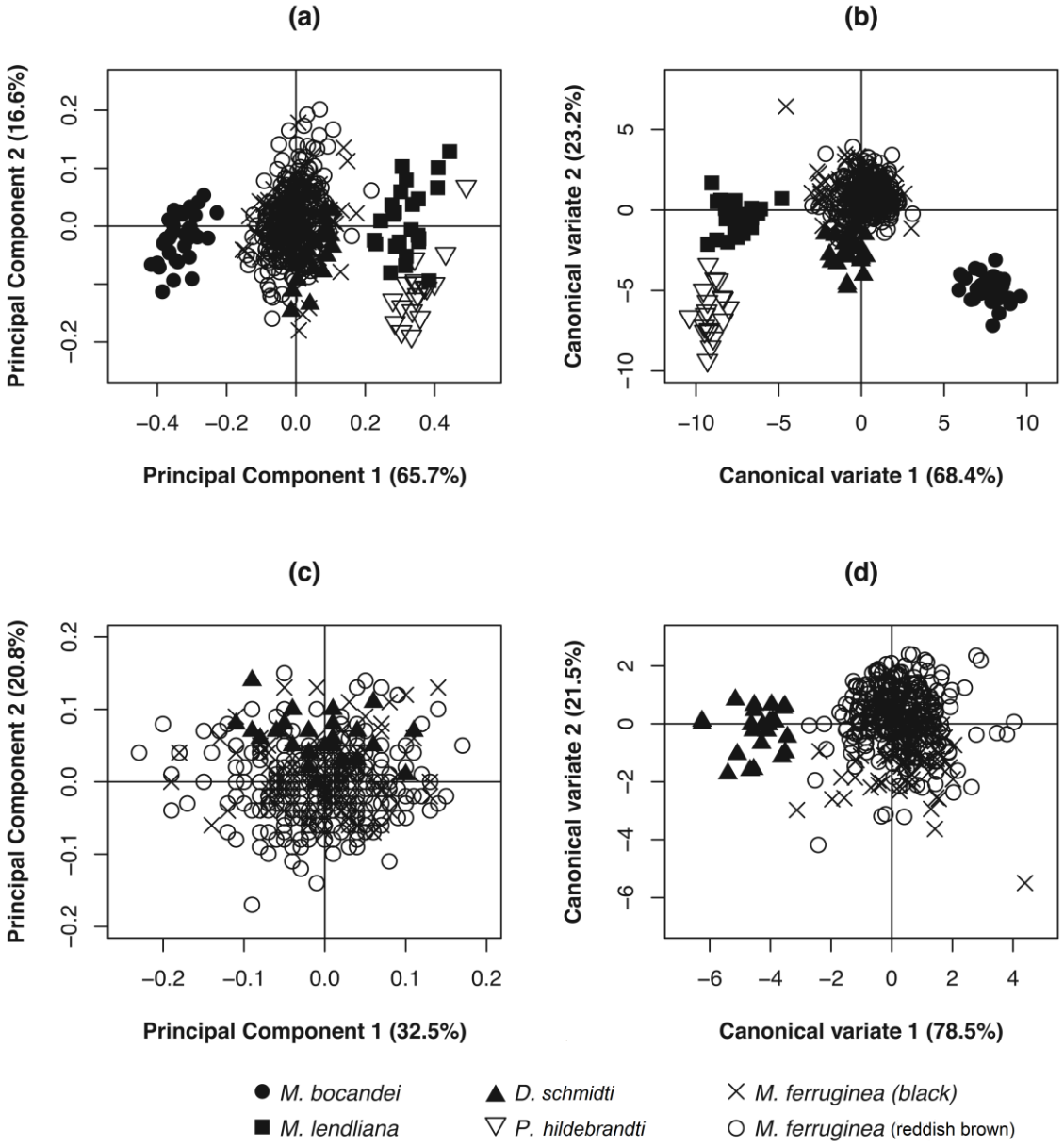

Figure 4 (a-d). Multivariate analyses of the wing morphometric measurements. (a). Principal component analysis (PCA) performed on the complete dataset with the 4 species and the 2 morphospecies. (b) Canonical variate analysis (CVA) performed on the complete dataset with the 4 species and the 2 morphospecies. (c) PCA performed on the measurement collected for *M. ferruginea* morphs and *D. schmidti*. (d) CVA performed on the measurement collected for *M. ferruginea* morphs and *D. schmidti*.

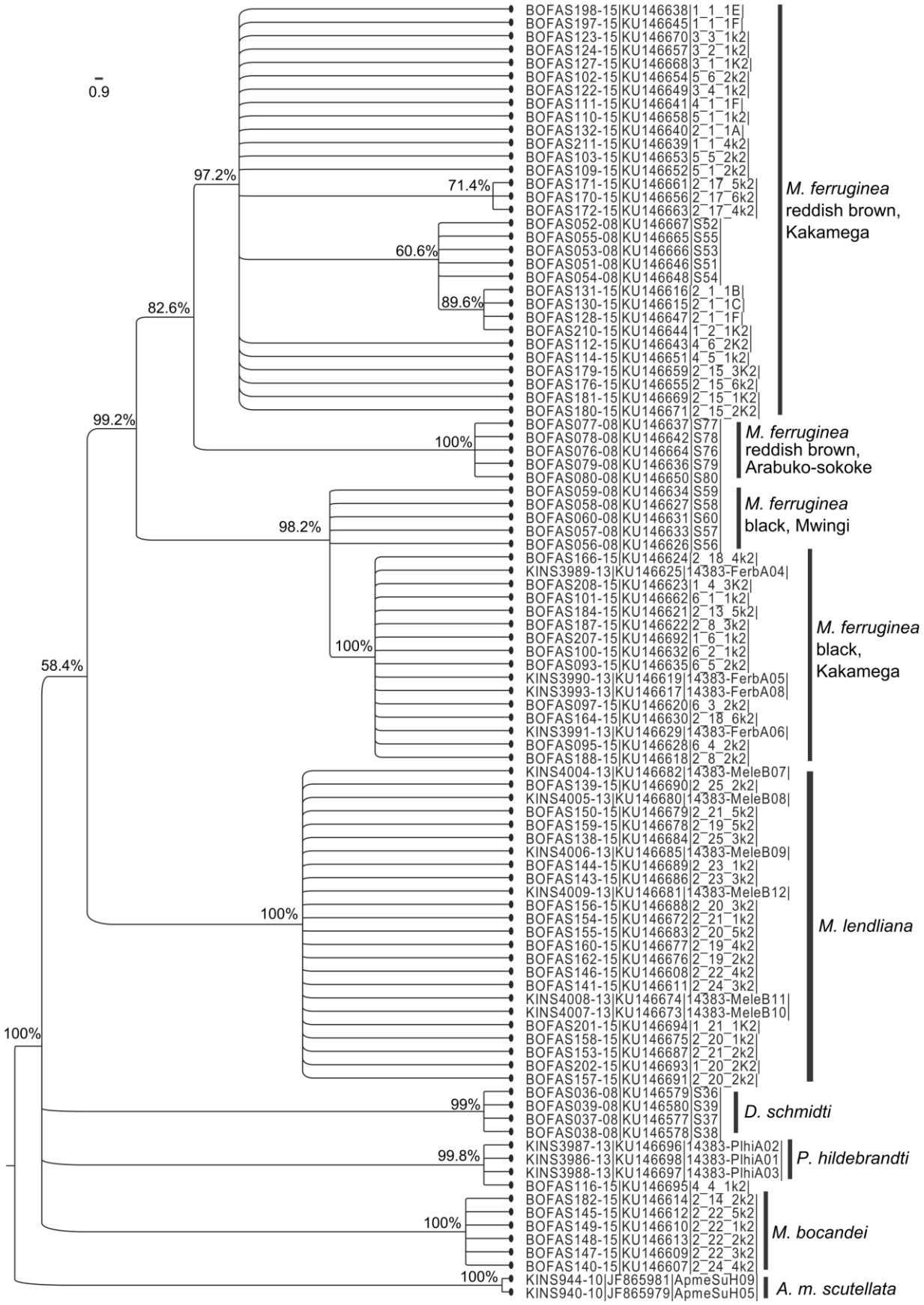

Figure 5. Maximum Likelihood phylogenetic tree generated using 95 *COI* sequences from five Kenyan stingless bees. Values on branches indicate bootstrap support values in percentages over 1000 replicates. Labels include the accession numbers of the BOFAS (Bees of the World—Africa - stingless bees) database which is part of BOLD (Barcode of Life database - www.barcodinglife.org). GenBank Accession numbers and sample IDs. *A. m. scutellata* is used as an out group to root the tree.

Figure 1S

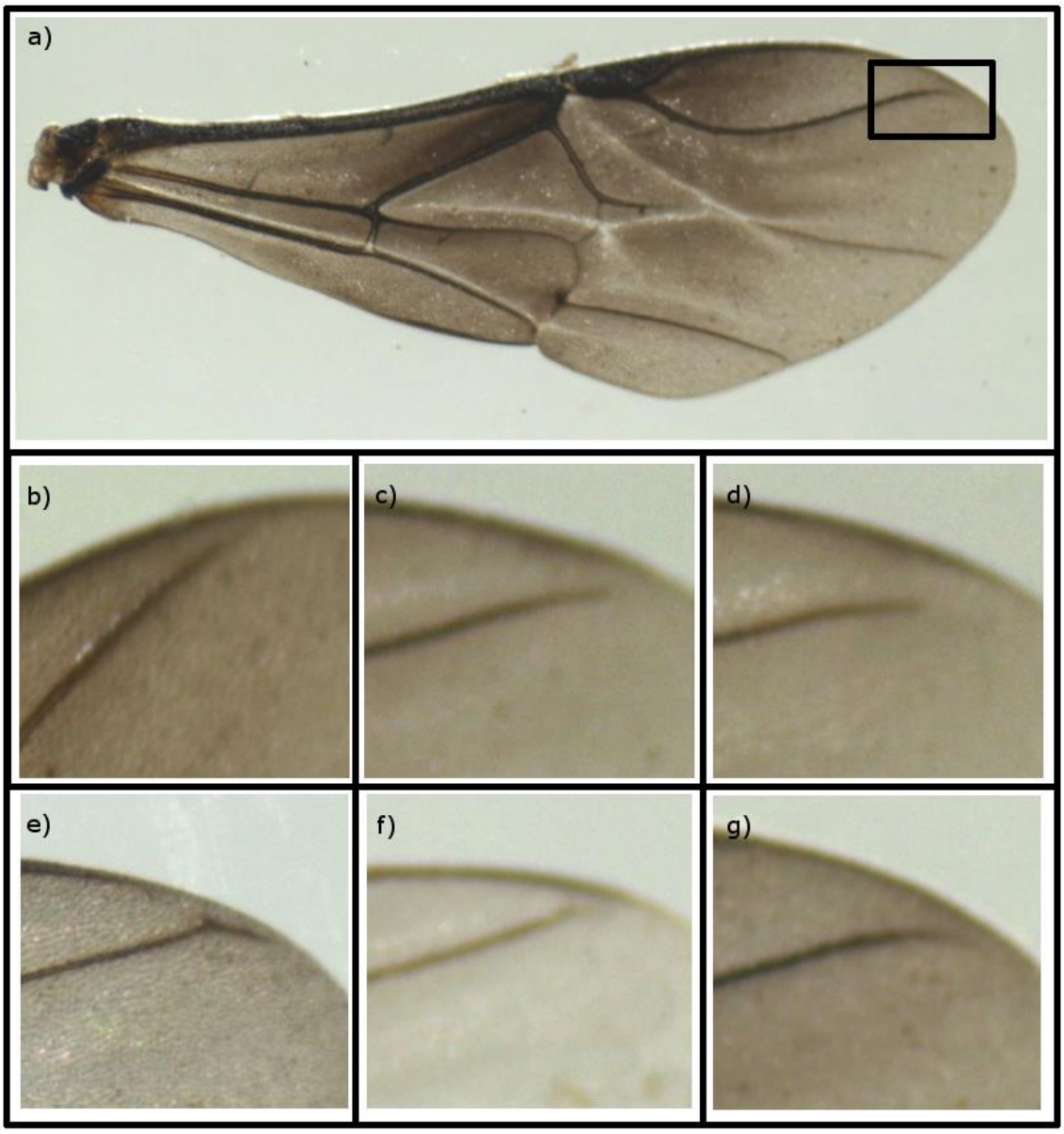

Figure 2S

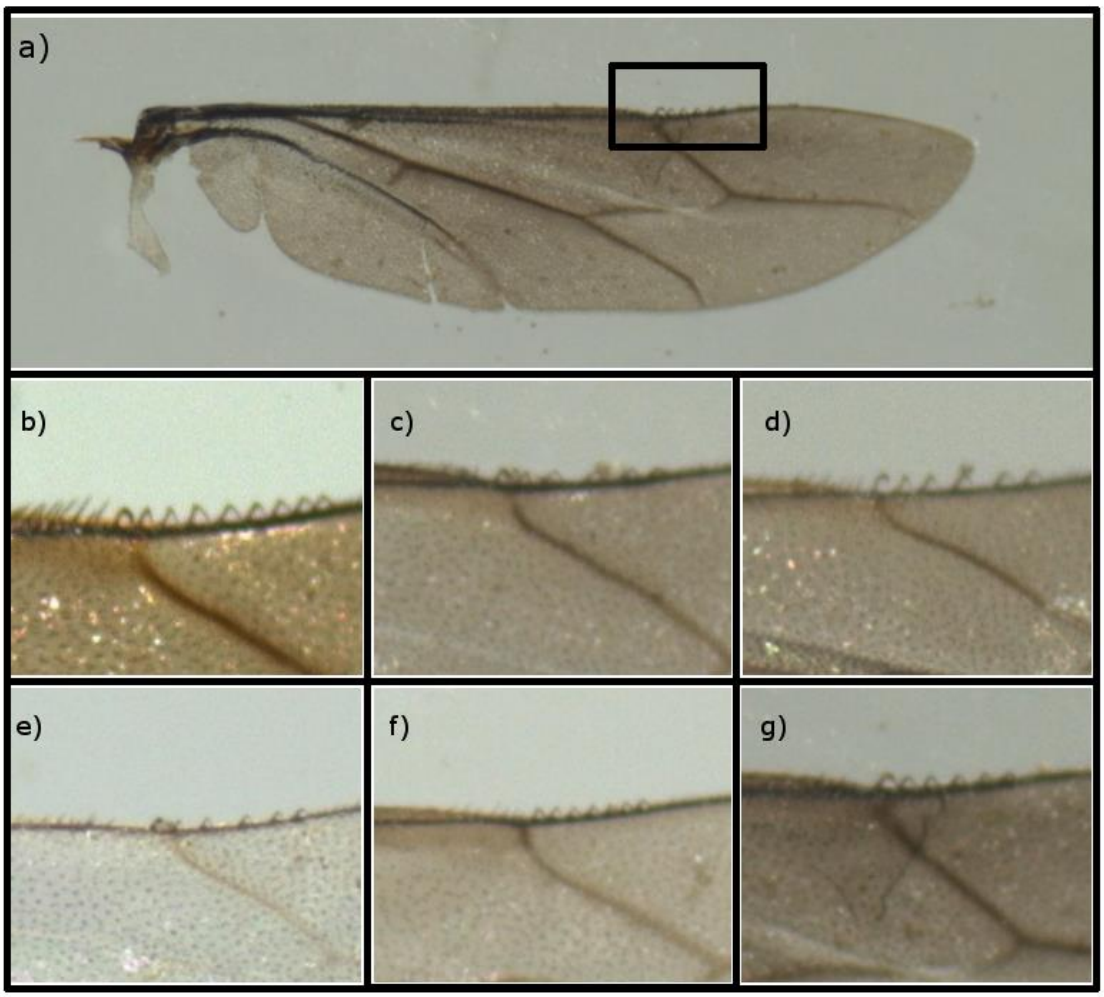

Figure 3S

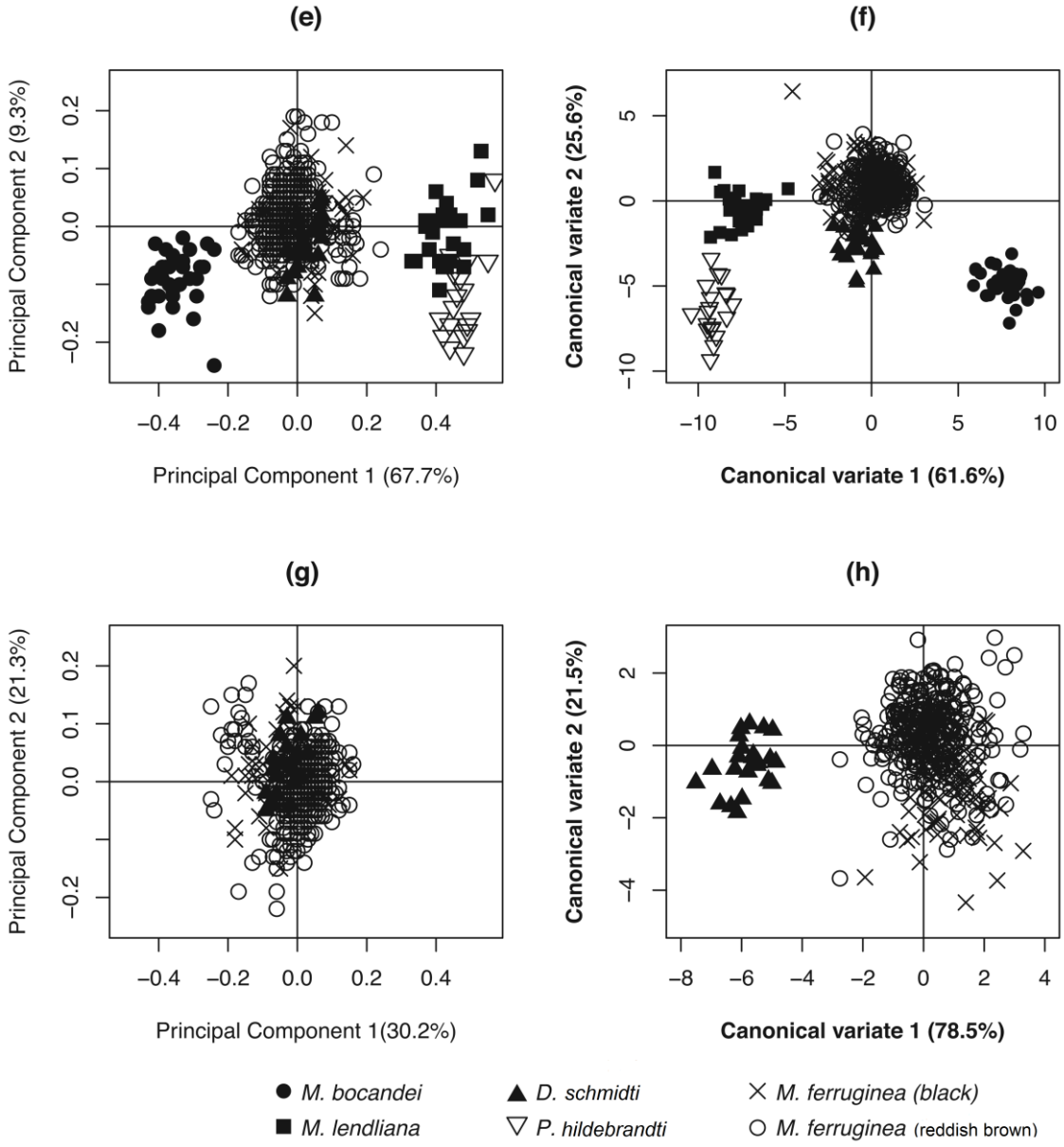
(e)
(f)
Principal Component 2 (9.3%)
Principal Component 1 (67.7%)
Canonical variate 2 (25.6%)
Canonical variate 1 (61.6%)
(g)
(h)
Principal Component 2 (21.3%)
Principal Component 1(30.2%)
Canonical variate 2 (21.5%)
Canonical variate 1 (78.5%)
M. bocandei
M. lendliana
D. schmidti
P. hildebrandti
M. ferruginea (black)
M. ferruginea (reddish brown)

Table 1S. Eigen values and coefficients (loadings) of the first two principal components (PC1 and PC2) for the log-transformed wing measurements data for the five and two stingless bee species (*M. ferruginea* and *D. schmidti*), respectively (considering the black and red *M. ferruginea* morphs separately).

	Five species		**Two species**	
	PC1	**PC2**	**PC1**	**PC2**
Proportion of variance	**65.7%**	**11.6%**	**32.5%**	**20.8%**
Eigen value	**0.1453**	**0.06105**	**0.06411**	**0.05121**
Variable	**Loadings**			
V1	-0.276	0.089	0.076	-0.114
V2	-0.285	0.043	0.102	-0.097
V3	-0.326	0.190	0.070	-0.183
V4	-0.266	-0.313	0.196	0.828
V5	-0.311	0.242	0.113	0.033
V6	-0.367	0.142	0.105	-0.277
V7	-0.323	-0.847	0.938	-0.026
V8	-0.346	0.083	0.102	-0.219
V9	-0.179	0.134	0.063	-0.146
V10	-0.377	0.181	0.129	-0.260
V11	-0.190	0.050	0.077	-0.208

Table 2S. Standardized canonical coefficient for canonical discriminant analysis on log-transformed wing measurement data for five and two species respectively (considering the black and red *M. ferruginea* morphs separately).

Standardized Canonical Coefficients				
	Five species		**Two species**	
Variables	**cv1**	**cv2**	**cv1**	**cv2**
V1	1.01	-4.99	-2.43	0.01
V2	0.78	-0.07	0.94	-0.08
V3	0.89	3.45	1.18	-0.52
V4	0.07	-0.26	-0.21	0.15
V5	0.55	1.27	0.51	-0.23
V6	0.73	1.09	1.01	1.29
V7	-0.01	-0.004	-0.07	-0.19
V8	0.01	-0.98	-0.62	0.18
V9	-0.61	0.99	0.34	-0.16
V10	0.14	0.25	0.25	0.35
V11	-0.19	-0.43	0.09	-0.25