Resolving taxonomic ambiguity and cryptic speciation of *Hypotrigona* species through Morphometrics and DNA barcoding

Nelly N Ndungu[a,b]*, Kiatoko Nkoba[a], Catherine L Sole[b], Christian WW Pirk[b], A Yusuf Abdullahi[b], Suresh K Raina[a] and Daniel K Masiga[a]

[a]International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya; [b]Social Insects Research Group, Department of Zoology and Entomology University of Pretoria, Pretoria, South Africa

Abstract

Stingless bees are important pollinators of cultivated and wild plants contributing significantly to biodiversity and food security. Conserving pollinator plant interactions is essential to secure these ecosystems services. The use of morphological features in the identification of stingless bees in the genus *Hypotrigona* is extremely difficult due to many similarities among species resulting in taxonomic ambiguity. Here, both traditional morphometrics and DNA barcoding were applied as complementary tools for the identification of three *Hypotrigona* species: *Hypotrigona gribodoi, H. ruspolii* and *H. araujoi.* The study results show that morphometrics separates *H. gribodoi* and *H. ruspolii* from *H. araujoi*; however there is an overlap between *H. gribodoi* and *H. ruspolii*. On the other hand, DNA barcoding separates the three species. There was lower genetic distance between *H. araujoi* and *H. gribodoi* from Kakamega (1.4%) than between *H. gribodoi* collected from Kakamega and *H. gribodoi* from Mwingi (4.3%). The high genetic distance or intraspecific distance within *H. gribodoi* strongly suggests cryptic speciation within this species, and that the *H. gribodoi* collected from Mwingi is a potentially new putative species. Thus the use of morphometrics and molecular taxonomic approaches (DNA barcoding) provide a convenient, robust and reliable way to identify *Hypotrigona* species. It also indicates the need for a thorough revision of *H. gribodoi* species.

Introduction

Stingless bees (Hymenoptera: Apidae: Meliponinae) are important pollinators of crops and wild plants (Heard, 1999; Kiatoko, Raina, Muli, & Mueke, 2014; Slaa, S´ anchez Chavesb, Chaves, Malagodi-Braga, & Hofstede, 2006) and are therefore a major compliment to honey bee pollination (Cortopassi-Laurino et al., 2006; Vanbergen, 2013). Furthermore, stingless bees have additional commercial features, which can be explored. Stingless bees produce honey that is different from that of honey bees due to its on average high moisture content, 31% and 20.2%, respectively, the high water content is due to enzymes and other substances that are associated with antibiotic activity of the stingless bee honey (Lubertus, Bruijn, Martens, & Sommeijer, 2006). Although stingless bee honey is produced in smaller quantities when compared to honey bees (*Apis mellifera*) (Kiatoko, Kumar, & Langevelde, 2016), their honey fetches higher prices due to its medicinal value (Kumar, Singh, & Alagumuthu, 2012). Stingless bee hive products include propolis and cerumen, all of which have been shown to have antioxidant activities (Pérez- Perez, Suárez, Pena_Vera, Gonzálvez, & Vit, 2013); antibacterial and immunomodulatory effect (Liberio et al., 2011; Temaru & Shimura, 2007); anti-inflammatory effects (Araujo, Libério, Guerra, Ribeiro, & Nascimento, 2012); inhibit dermal carcinogenesis in rodents (Pereira-Filho et al., 2014) and thus could be utilized in medicine. These commercial opportunities, combined with their ability to pollinate important plants have led to an increased interest in their commercial cultivation. The exploitation is however limited by lack of basic biological knowledge and the ability to easily distinguish species. Furthermore, taxonomic clarity is paramount for understanding pollinator ecology, especially in understudied areas like Africa (Archer, Pirk, Carvalheiro, & Nicolson, 2014).

Stingless bee species are grouped into two tribes: Trigonini and Meliponini that occur in Tropical and Neotropical regions of the world, respectively (Michener, 2000; Wille, 1983). They are differentiated from other bees by reduced sting and wing venation and the presence of penicillium on the hind tibiae (Eardley, 2004, Michener, 2007). However, the penicillium is absent or much reduced and soft in the genera *Hypotrigona* and *Cleptotrigona* (Eardley, 2004). In Africa, 19 species in six genera have been identified (Eardley 2004); these include *Dactylurina* Cockerell, 1934a, *Meliponula* Cockerell, 1934, *Plebeina* Moure, 1961a, *Hypotrigona* Cockerell, 1934a, *Liotrigona* Moure, 1961a and *Cleptotrigona* Moure, 1903 (Eardley, 2004). *Cleptotrigona* workers are known to rob pollen and nectar from other stingless

bees while workers from the remaining genera collect their own food from wild flowers and commercial crops (Eardley, 2004). *Hypotrigona,* the focus of this study, contains four species, *H. gribodoi, H. araujoi, H. ruspolii* and *H. penna*, of which the last one occurs in West Africa.

African Stingless bees have been poorly studied, with the result that classification of the group is still largely unresolved (Eardley, 2004; Michener, 2000). *Hypotrigona* species are known to be especially difficult to identify due to the similarity in their body morphology (Eardley, 2004). Several studies have attempted to distinguish the three East African *Hypotrigona* species: for instance Guiglia (1955) described *H. gribodoi* morphologically*;* Michener (1959) confirmed through breeding experiment that *H. araujoi* and *H. gribodoi* could not mate and were indeed biological species. Moure (1961) separated *H. gribodoi* and *H. araujoi* based on whole body length ratio, and Eardley (2004) generated a taxonomic key in which he showed that the character differentiating the three *Hypotrigona* species is that in *H. ruspolii* there is an imaginary line posterior to midline of the hind tibia while in *H. gribodoi* and *H. araujoi* the line is in the middle. In addition, Eardley (2004) used the worker legs, wings, head and thorax to describe *Hypotrigona* species where, head and scutal vestiture weakly pinnate and scutal punctuation is slightly shiny. Despite these attempts to differentiate *Hypotrigona* species, it still remains difficult to identify these species without the expert taxonomic knowledge needed to interpret the keys. Tools that are easier to use are needed to differentiate between *Hypotrigona* species that can be applied both at a large scale and to varying levels of taxonomic expertise.

This study therefore combines morphometrics and DNA barcoding in an attempt to identify and differentiate the closely related species of *Hypotrigona* in Kenya. DNA barcoding tools have been used previously to identify bees; (Hurtado-Burillo, Ruiz, De Jesús May-Itzá, Quezada-Eúan, & De La Rúa, 2013; Magnacca & Brown, 2012; Sheffield & Hebert, 2009) and ants in the genus *Solenopsis* (Delsinne et al., 2016). A new species of sweat bee, *Lasioglossum ephialtum (Gibbs)* was described using DNA barcoding, in combination with geographical and morphological data (Gibbs & Dumesh, 2013). Recently five stingless bee species in Kenya have been identified using morphometrics and DNA barcoding, revealing cryptic speciation within the *Meliponula ferruginea* reddish brown and black "morphospecies" (Ndungu et al., 2017). The 5' end of *CO1* was chosen as the focal region because it is bordered by two universal primers that work for a wide range of metazoans (Hebert, Cywinska, Ball, & deWaard, 2003; Puillandre et

al., 2012) and has been shown to be most informative for species identification (Hajibabaei, Singer, Hebert, & Hickey, 2007; Sheffield & Hebert, 2009). The aims of this chapter therefore were to apply a DNA barcoding protocol based on the cytochrome c oxidase 1 (*COI*) gene sequence and morphometric analysis to identify the species of *Hypotrigona*.

Methodology

Study sites

Stingless bee samples were collected from 2014 to 2015 across two ecological zones in Kenya, namely Kakamega and Mwingi which are geographically distant and cover high and medium altitudes, respectively (Fig. 1.). Kakamega forest is a tropical rain forest in western Kenya (latitude 0°09'N to 0°22'N and longitude 34°50'E to 34°58'E), supporting high biodiversity (Zimmerman, 1972) including bees (Kasina, Mburu, Kraemer, & Holm-Mueller, 2009; Nkoba, Raina, Muli, Mithofer, & Mueke, 2012). Mwingi is an arid to semi- arid region in eastern Kenya (0°51′S, 38°22′E) that lies between 600 - 900 m above sea level (Njoroge, Kaibui, Njenga, & Odhiambo, 2010).

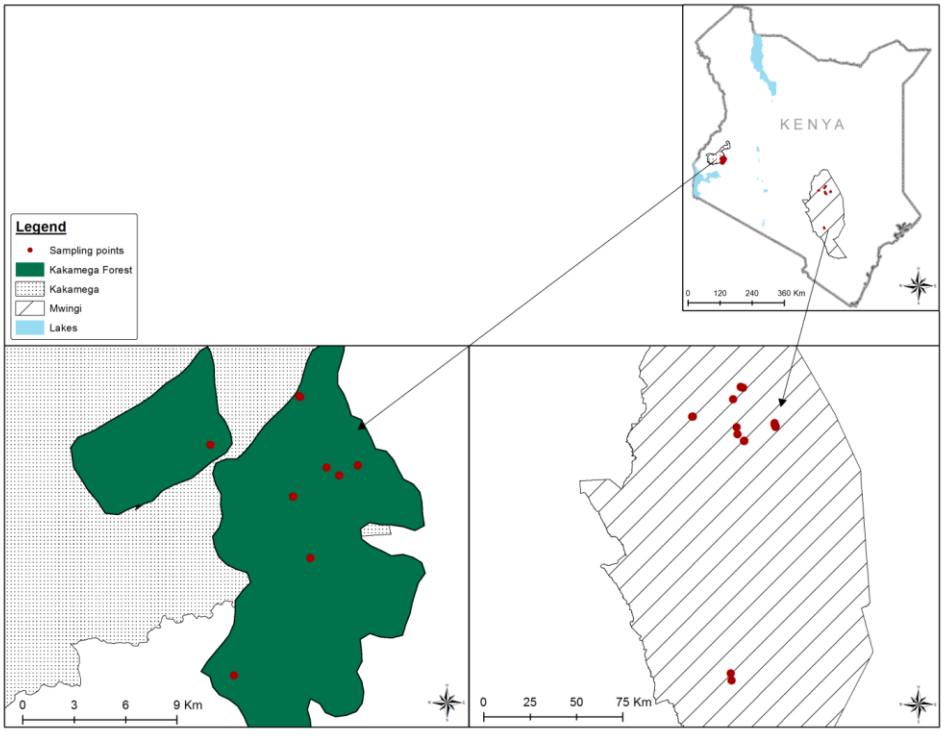

Fig. 1 Map of Kenya showing the two sampling areas, Kakamega forest (green) and Mwingi (chequered box). Circles represent all sampling points

Sampling method

A total of 163 samples were collected for morphometric analysis. The number of sampled colonies varied across species and sites depending on availability as follows: *H. ruspolii* from Kakamega (17 colonies); *H. araujoi* from Kakamega (6 colonies); *H. gribodoi* from Mwingi (25 colonies) and *H. gribodoi* from Kakamega (26 colonies). As *H. penna* occurs in West Africa and could not be collected, this study only focuses on three of the species. The samples collected from both sites were used for morphometrics and DNA barcoding.

Morphometrics

Stingless bees were dissected under the microscope to remove the right forewing and right hind leg. The legs and wings were mounted on 2 mm slides and images taken using a Leica EZ4D stereomicroscope (Leica Microsystems Limited, Germany). Measurements were taken using the microscope accompanying software LAS EZ, version 1.4.0. Eight wing and three leg morphometric characters were selected for measurement in accordance with previous studies (Hartfelder & Engels, 1992; J. Quezada-Euán et al., 2007). Each measurement was taken in triplicate (to an accuracy of 0.001 mm). Measurements included forewing length (WL), forewing width (WW), distances between selected forewing veins, V3–V8, and tibia length (TL), tibia width (TW), and femur length (FL) (Fig. 2 A and B). Voucher specimens are preserved at the museum of the African Reference Laboratory for Bee Health *icipe* in Nairobi, Kenya.

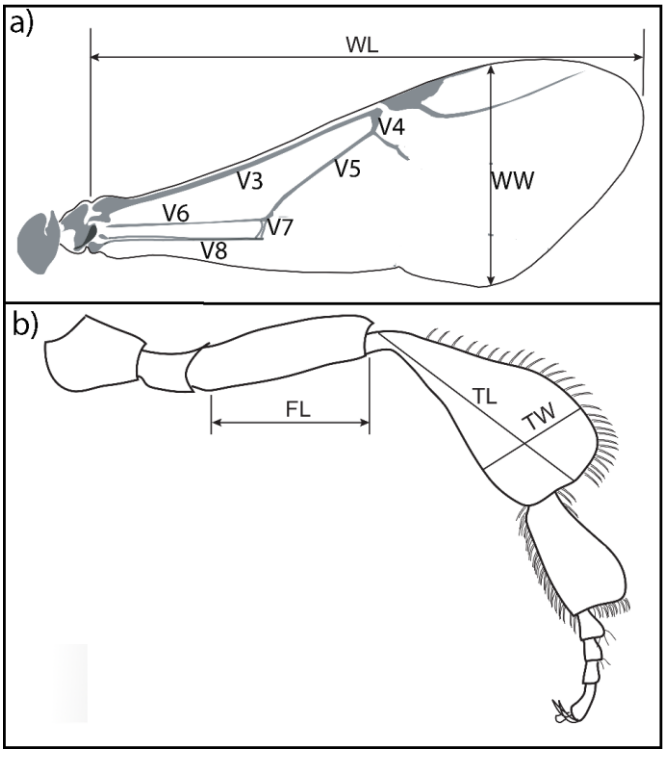

Fig. 2 Schematic representation of the right forewing and the right hind leg presenting morphometric characters of interest. (a) Right forewing showing veins used in morphometrics studies. WL= wing length; WW= wing width; V3= Marginal vein (R); V4= radial sector (RS); V5 = basal vein (M); V6= medial–cubital vein (M+Cu); V7= cubitus (Cu); V8= V. (b) Right hind leg of a stingless bee. FL= femur length; TL= tibial length; TW= tibial width

DNA extraction, amplification of the barcoding region and sequencing

Genomic DNA was extracted from individual stingless bee legs using an Isolate II genomic DNA extraction Kit (Bioline) in a final elution volume of 30 µl. DNA barcoding procedure followed the Barcode of Life Database (BOLD) recommendations, thus we made sure that at least three DNA barcodes were sequenced to represent each species (Ratnasingham & Hebert, 2007). The extracted DNA was stored at –20 $^{\circ}$C until required for amplification. The universal primer pair forward primer LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse primer HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (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 volume of 25 µl containing 10 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 50 ng/µl 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 using ethidium bromide on a 1.2% agarose gel. 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 GenBank (Appendix I Table 1).

Data Analyses

Multivariate analyses of morphometrics

Morphometric analyses were performed using R 3.2.1 (R Development Core Team, 2015). Principal Component Analysis (PCA), a multivariate method that does not assume a priori grouping of individuals was used to determine the clustering of different species. Data were log transformed (log_{10}) before analysis to conform to the assumptions of PCA (Keene, 1995). The first and second Eigen values were considered in the interpretation of the PCA output, as they were associated with the majority (>70%) of the variation between samples. Character loadings were obtained for the first two principal components, to provide an indication of the influence of each character on the principal components. The first two principal component scores were plotted for forewing and leg measurements. The log-transformed data were also subjected to Canonical Variate Analysis (CVA) to analyze group structure in the multivariate data. In addition, Mahalanobis squared distances (D^2) between species were computed across morphometric characters. Mahalanobis squared distance (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). Mahalanobis squared distance was calculated to complement PCA and CVA plots, and the genetic distances.

Analysis of COI sequence data

BOLD Analysis Tools

Barcode of Life Data systems (BOLD) workbench tools were used to generate various results that include: sequence base composition, diagnostic characters (differences in base pairs i.e. characters), Barcode gap analysis and distance summary (http://www.boldsystems.org/). To generate diagnostic characters, the sequences of *Hypotrigona* species were aligned using Muscle and the positions at which the nucleotides differ were used as diagnostic characters. The

diagnostic character analysis provides a means to examine nucleotide polymorphism between consensus sequences of the *Hypotrigona* species and characterizes how unique the consensus bases are compared to the other consensus sequences. To determine the distribution of distances within each species and the distance to the nearest neighbor of each species, the Barcode Gap Analysis was done using Kimura-2 parameter distance model and MUSCLE (Edgar, 2004) alignment option. Barcode Gap analysis is the distance to the nearest neighbor for the species. Lastly, to report the sequence divergence between barcode sequences at the species level and within species divergence, distance summary was calculated using the BOLD tools (Ratnasingham & Hebert, 2007).

Phylogenetic Analyses

Bioedit (Hall, 1999) was used to assemble and edit the sequences, and alignment done using Muscle, (Edgar, 2004), in MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) with default settings and then converted into Phylip format using Seaview (Gouy, Guindon, & Gascuel, 2010). To view the separation of *Hypotrigona* species, phylogenetic trees were deduced using criteria for Maximum likelihood (ML) as implemented in RAxML v8.2.0 (Stamatakis, 2014) and neighbor-joining (NJ) as implemented in MEGA 6.

For ML analyses COI was assigned a GTR + G model and empirical base frequencies were estimated by the program, while for the NJ method p-distance was used to estimate the phylogeny. For both analyses node support was estimated by non-parametric bootstrap (Felsenstein, 1995) based on 1000 replicates. In addition, to calculate genetic distances, pairwise genetic distances (p-distance) within and between species were calculated in MEGA 6. Two *Meliponula lendliana* from the BOFAS project in BOLD were used as out groups (KU146611 and KU146608).

Results

Morphometrics

In the PCA plot, *H. ruspolii* separated completely from *H. araujoi*; however, *H. gribodoi* overlaps with *H. ruspolii* and *H. araujoi*. *Hypotrigona gribodoi* from Mwingi and *H. gribodoi* from Kakamega formed a single cluster. PC1 and PC2 contributed 64.4% and 13.4% respectively to the total variation in separation of the species (Fig. 3a). Tibia width and length showed the

highest contribution in PCA 1 to the differentiation of the species (0.5 and 0.417, respectively). Separation was greater in a CVA plot that sought to find maximal differences among a priori defined groups; CV1 and CV2 accounted for 78.9% and 16.7% of the variance in the data, respectively (Fig. 3b). Three clusters were formed; a) *H. araujoi* alone, b) *H. gribodoi* from Mwingi partially separated from *H. gribodoi* from Kakamega and c) *H. ruspolii*. Mahalanobis squared distance (D^2) shows that the largest distance is between *H. araujoi* and *H. ruspolii* (44.65), while the shortest distance was between *H. gribodoi* from Kakamega and *H. gribodoi* from Mwingi (9.47). Mahalanobis squared distance between *H. araujoi* and *H. gribodoi* from Kakamega was larger than D^2 between *H. araujoi* and *H. gribodoi* from Mwingi (21.83 and 12.83), respectively.

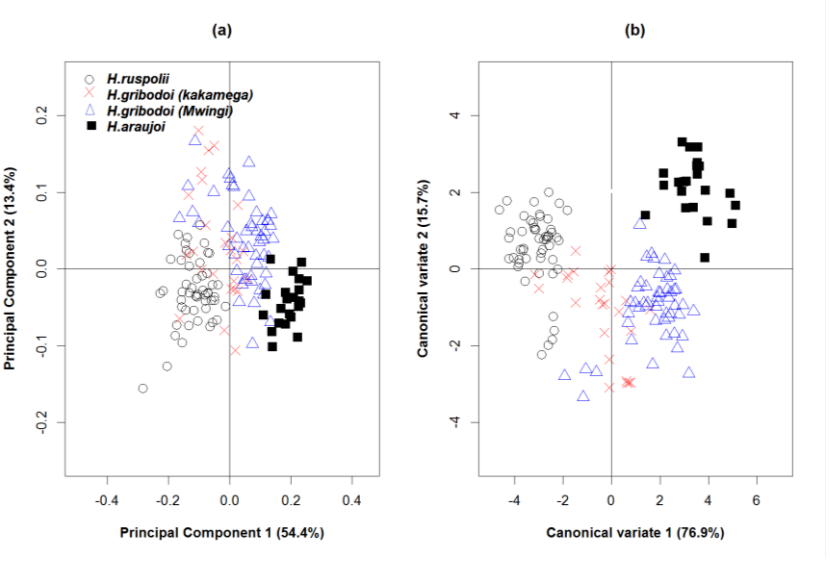

Fig. 3 Multivariate analyses of the wing morphometric measurements. (a). Principal component analysis (PCA) performed on *Hypotrigona* species. PC1 and PC2 contributed 67.8% (54.4% and 13.4%) respectively in the separation. There was partial separation of *H. araujoi* from *H. gribodoi* and *H. ruspolii* (b) Canonical variate analysis (CVA) performed on the complete dataset with the *Hypotrigona* species. CV1 and CV2 contributed 93.6% to the separation (76.9% and 16.7%) respectively. *Hypotrigona araujoi* and *H. ruspolii* separate completely. There was an overlap between *H. ruspolii* and *H. gribodoi* from Kakamega

Analysis of COI sequences

BOLD Analysis

Hypotrigona ruspolii had 25 diagnostic characters, while *H. araujoi* and *H. gribodoi* had 25 and 9 partial diagnostic characters, respectively (Fig. 4). In terms of Barcode Gap Analysis, the mean intraspecific distance within each species is 1.46 ± 0.19% while mean distance to the Nearest Neighbor (NN) is 2.67 ± 1.04%. The highest intraspecific distance was observed in *H. gribodoi* from Mwingi and *H. gribodoi* from Kakamega (5.41%), followed by *H. araujoi* and *H. ruspolii* 2.66% and 2.51%, respectively (Table 1). BOLD calculated genetic mean distance within species and the genus were 1.76% and 7.08%, respectively.

Table 1. DNA Barcode Gap Analysis of the three *Hypotrigona* species

Species	Mean Intraspecific (%)	Maximum Intra specific (%)	Nearest Species	Distance to Nearest Neighbor(NN) (%)
H. araujoi	1.76	2.66	*H. gribodoi*	0.46
H. gribodoi	1.95	5.41*	*H. araujoi*	0.46
H. ruspolii	0.67	2.51	*H. gribodoi*	7.08

Sequence divergence for all sequences compared at the species and genus level. * *H. gribodoi* from Kakamega and *H. gribodoi*, Mwingi combined

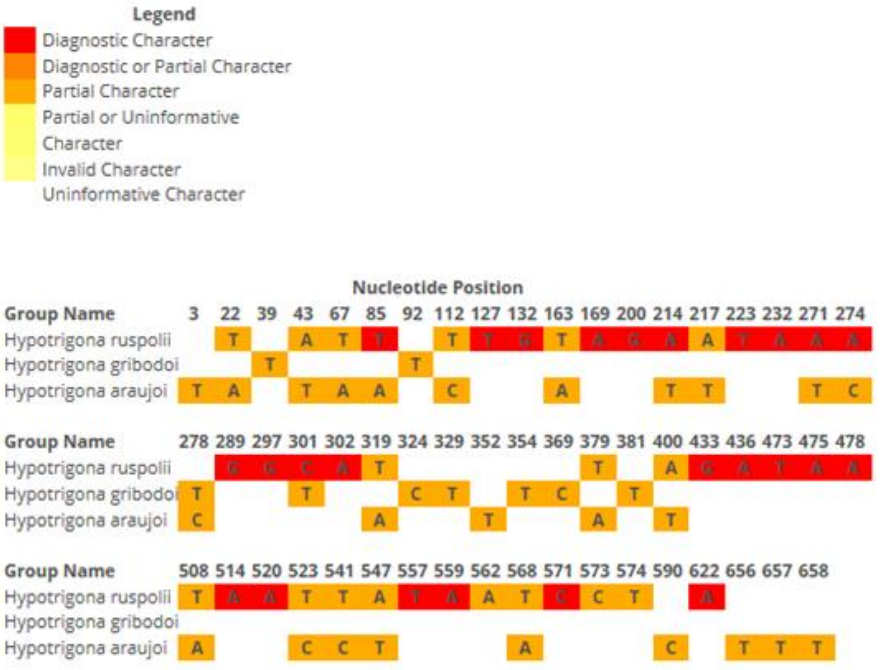

Fig. 4. Characters that differentiate *H. gribodoi*, *H. ruspolii* and *H. araujoi*, generated with BOLD analysis tools.

The Individual sequence length = 658. No. groups in MSA with minimum 3 sequences = 3. Legend: *= diagnostic character; P= partial character. *Hypotrigona ruspolii* was the only species with specific diagnostic characters 25 and 17 partial diagnostic characters, while *H. araujoi* and *H. gribodoi* had 25 and 9 partial diagnostic characters respectively.

Phylogenetic and distance analysis

The maximum likelihood and Neighbor Joining methods supported the monophyly of *Hypotrigona* (99/100% bootstrap support, respectively) (Fig. 5). *Hypotrigona ruspolii* is a well-supported monophyletic species (99/100% bootstrap support, respectively) and is sister to all the other species. *H. gribodoi* from Kakamega and *H. araujoi* form a paraphyletic clade (99/98% bootstrap support, respectively) and are sister to *H. gribodoi* (Mwingi) (93/79% bootstrap support, respectively). *Hypotrigona gribodoi* from Mwingi forms a monophyletic albeit poorly supported clade (58/-% bootstrap support; respectively). Based on genetic distance, *H. araujoi* appears more closely related to *H. gribodoi* from Kakamega with a distance of 0.015 (1.5%) and more distant from *H. gribodoi* from Mwingi at 0.061 (6.1%). The distance between *H. gribodoi* from Mwingi and *H. gribodoi* from Kakamega was 0.043 (4.3%). The highest genetic distance observed was between *H. araujoi* and *H. ruspolii*, 0.107 (10.7%). The highest within group mean

Fig. 5. RAxML phylogram, bootstrap values for both Maximum Likelihood (ML) and Neighbor-joining (NJ) analyses are displayed above the nodes (NJ/ML). 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

distance was in *H. araujoi* at 0.017 (1.7%), followed by *H. gribodoi* from Mwingi at 0.0084 (8.4%) and lastly *H. gribodoi* from Kakamega at 0.0021 (0.21%) (Fig 5).

Discussion

Morphometrics has been used in a number of studies aimed at differentiating species and races of honey bees (*Apis mellifera*) (Quezada-Euán et al., 2011; Raina & Kimbu, 2005) and bumblebees (Owen, 2009). *Hypotrigona gribodoi* from Kakamega overlaps with *H. ruspolii* and *H. gribodoi* from Mwingi (Fig. 3a and b). Overlap is expected since *Hypotrigona* species are very similar in morphological features and all have a small body size (~2mm) (Eardley 2004).

Reduced and weak wing venation were observed in all *Hypotrigona* species, a characteristic common in stingless bees (Wille, 1983). The results show that tibia width and length showed the highest contribution to the differentiation of the species. These results are supported by Eardley (2004), where *H. ruspolii* was shown to have the smallest tibia in terms of width amongst the *Hypotrigona* species (Eardley, 2004). These results corroborate a previous study where *H. araujoi* had been shown to have a wider tibia compared to *H. gribodoi* ((Michener, 1959). In addition, Eardley (2004) also reported that *H araujoi* is the largest of the *Hypotrigona* species while *H. ruspolii* is the smallest in terms of body size. Therefore, tibial length and width can be used to differentiate *H. ruspolii* and *H. araujoi*.

DNA barcoding results contrast with those of the morphometric analyses as *H. ruspolii* is a well-supported monophyletic clade separate from *H. gribodoi* and *H. araujoi*. There are 25 diagnostic characters that can be used efficiently to separate *H. ruspolii* from other *Hypotrigona* species. The separation of *H. ruspolii* from *H. araujoi* in the CVA plots is supported by DNA barcoding results where the two separate with the highest genetic distance (10.3%). Thus, there is a strong indication that *H. ruspolii* is genetically distant from the other *Hypotrigona* species. On the other hand, only partial diagnostic characters were observed for *H. gribodoi* and *H. araujoi* thus the two species are more difficult to differentiate within the *Hypotrigona* species.

In addition, there was lower interspecifc distance between *H. araujoi* and *H. gribodoi* from Kakamega when compared to *H. gribodoi* from Mwingi and *H. gribodoi* from Kakamega. *Hypotrigona gribodoi* collected from Kakamega forms a paraphyletic clade with *H. araujoi* and therefore appears more closely related than *H. gribodoi* from Mwingi and Kakamega. *Hypotrigona gribodoi* and *H. araujoi* were previously considered a single species due to high

morphological similarity. However, Araujoi and Kerr (1959) in their study in Luanda, Angola, reported that *H. gribodoi* and *H. araujoi* do not interbreed and are thus different species. In addition to this they differ in nest architecture, cluster arrangement and horizontal combs. Araujo and Kerr (1959) termed *H. gribodoi* and *H. araujoi* as cryptic or sibling species, which is evident from the molecular data for samples collected in Kakamega Forest (Fig. 5). Such results have been observed in butterflies where closely related but morphologically and ecologically distinct species differed by only one to three nucleotides (Burns, Janzen, Hajibabaei, Hallwachs, & Herbert, 2007). The high intraspecific variation within *H. gribodoi* from Mwingi and *H. gribodoi* from Kakamega could be a result of adaptation to different environments and they may represent independent evolutionary units. Such high genetic distance was found in a stingless bee *Plebeia remota* where the samples were collected from two different localities in Brazil whose ecological characteristics differed significantly and, thus, it was suggested that paleogographic and paleoclimatic events led to isolation of the two populations (Cristina, Magalhães, & Oliveira, 2006). The morphometric-based PCA and CVA analyses revealed an overlap and partial separation of *H. gribodoi* from Mwingi and *H. gribodoi* from Kakamega; thus, in terms of size, we suggest that the two represent different species that are cryptic (i.e. morphologically indistinguishable).

DNA barcoding separated the three *Hypotrigona* species completely and can therefore be reliably used for species identification. The low genetic distance between *H. araujoi* and *H. gribodoi* from Kakamega shows that the two species are closely related. However, using mophometric tools, the two species separated completely in the CVA. The results indicate the need for integration of morphometrics and DNA barcoding. Integration of morphometric and DNA barcoding tools have been used in a study of the stingless bee *Melipona yucatanica* to detect cryptic speciation (May-Itzá, Quezada-Euán, Medina, Enríquez, & de la Rúa, 2010), to resolve the taxonomy of western Malagasy stingless bee *Liotrigona moure* (Koch, 2010) and for the differentiation in the Neotropical bee *Melipona beechii* (Quezada-Euán et al., 2007). Reliable identification requires combining DNA barcoding and the morphometrics as tools for differentiating the three *Hypotrigona* species.

The data suggest a likelihood of cryptic speciation within *H. gribodoi* species and thus a potentially new putative species of *H. gribodoi* collected from Mwingi. Mwingi and Kakamega are highly diverse in terms of climatic conditions and geographic distance, potential reasons for

high intraspecific genetic distances observed (Cristina et al., 2006). Kakamega forest is located in the highlands of western Kenya and is a tropical rainforest that lies between 1500 - 1600 m above sea level (Tsingalia & Kassily, 2009) with an average annual rainfall of 1200 – 1700 mm. Mwingi, on the other hand, is a mid-altitude and semi- arid area that lies between 600 - 900 m above sea level (Njoroge et al., 2010). The climate is hot and dry across most of the year with an average annual rainfall of 400 – 800 mm and temperatures that vary throughout the year, ranging between 24 - 34ºC (Njoroge et al., 2010; Opiyo, Mureithi, & Ngugi, 2011). Large areas are occupied by grasslands and shrubs, mainly consisting of dry land vegetation (Kaloi, Tayebwa, & Bashaasha, 2005).

The *H. gribodoi* populations in Mwingi and Kakamega are isolated by a large geographical distance of approximately 500 km which includes the Great Rift Valley (https://en.wikipedia.org/wiki/Great_Rift_Valley, _Kenya) and thus interbreeding between these two *H. gribodoi* populations is unlikely. A study carried out on *Melipona subtinida* showed high intraspecifc variation, which was taken as an evidence of isolation (Cruz et al., 2006). It has been suggested that stingless bees migrate for short distances of about fifty to few hundred meters between conspecific colonies (Roubik, 2006). More specifically, *Hypotrigona* species mate about 100m around their nests (Portugal-Araujo & Kerr, 1959) and they are known to forage across short distances of about 300m (Wille, 1983).

In conclusion, integration of morphometrics and DNA barcoding has successfully identified and differentiated the three *Hypotrigona* species. The study suggests adopting DNA barcoding and morphometrics to identify *Hypotrigona* species. The high genetic distance or intraspecific distance within *H. gribodoi* suggests the possibility of cryptic speciation and thus a potentially new putative species should be described. Additional

References

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APPENDIX I

Table 1. BOLD, Sample ID and GenBank Accession numbers for all the samples used in this work

Identification	BOLD	Sample ID	GenBank Accession
Hypotrigona araujoi	BOFAS282-15	31_2K3	KU567204
Hypotrigona araujoi	BOFAS272-15	21_2K3	KU567207
Hypotrigona araujoi	BOFAS285-15	34_2K3	KU567212
Hypotrigona araujoi	BOFAS284-15	33_2K3	KU567210
Hypotrigona araujoi	BOFAS283-15	32_2K3	KU567208
Hypotrigona araujoi	BOFAS271-15	19_2K3	KU567203
Hypotrigona gribodoi	BOFAS281-15	30_2K3	KU567209
Hypotrigona gribodoi	BOFAS049-08	S49	KU146599
Hypotrigona gribodoi	BOFAS230-15	2_1_1_1m	KU146592
Hypotrigona gribodoi	BOFAS047-08	S47	KU146584
Hypotrigona gribodoi	BOFAS014-08	S14	KU146585
Hypotrigona gribodoi	BOFAS175-15	2_16_5k2	KU146587
Hypotrigona gribodoi	BOFAS273-15	22_2K3	KU567206
Hypotrigona gribodoi	BOFAS244-15	4_17_6m	KU567271
Hypotrigona gribodoi	BOFAS023-08	S23	KU567272
Hypotrigona gribodoi	BOFAS011-08	S11	KU567274
Hypotrigona gribodoi	BOFAS061-08	S61	KU567213
Hypotrigona gribodoi	BOFAS027-08	S27	KU567214
Hypotrigona gribodoi	BOFAS020-08	S20	KU567215
Hypotrigona gribodoi	BOFAS019-08	S19	KU567216
Hypotrigona gribodoi	BOFAS009-08	S09	KU567217
Hypotrigona gribodoi	BOFAS134-15	2_28_2k2	KU567218
Hypotrigona gribodoi	BOFAS006-08	S06	KU567221
Hypotrigona gribodoi	BOFAS232-15	2_1_1_5m	KU567223
Hypotrigona gribodoi	BOFAS017-08	S17	KU567224
Hypotrigona gribodoi	BOFAS003-08	S03	KU567225
Hypotrigona gribodoi	BOFAS024-08	S24	KU567228
Hypotrigona gribodoi	BOFAS007-08	S07	KU567229
Hypotrigona gribodoi	BOFAS018-08	S18	KU567230
Hypotrigona gribodoi	BOFAS064-08	S64	KU567234
Hypotrigona gribodoi	BOFAS032-08	S32	KU567235
Hypotrigona gribodoi	BOFAS005-08	S05	KU567236
Hypotrigona gribodoi	BOFAS050-08	S50	KU567238
Hypotrigona gribodoi	BOFAS248-15	5_1_3m	KU567239
Hypotrigona gribodoi	BOFAS033-08	S33	KU567240
Hypotrigona gribodoi	BOFAS002-08	S02	KU567241
Hypotrigona gribodoi	BOFAS035-08	S35	KU567242
Hypotrigona gribodoi	BOFAS065-08	S65	KU567243
Hypotrigona gribodoi	BOFAS031-08	S31	KU567244
Hypotrigona gribodoi	BOFAS025-08	S25	KU567246
Hypotrigona gribodoi	BOFAS034-08	S34	KU567247
Hypotrigona gribodoi	BOFAS010-08	S10	KU567248
Hypotrigona gribodoi	BOFAS028-08	S28	KU567251
Hypotrigona gribodoi	BOFAS063-08	S63	KU567252
Hypotrigona gribodoi	BOFAS008-08	S08	KU567253

Hypotrigona gribodoi	BOFAS001-08	S01	KU567254
Hypotrigona gribodoi	BOFAS213-15	1_1_5k1	KU567256
Hypotrigona gribodoi	BOFAS242-15	4_17_3m	KU567258
Hypotrigona gribodoi	BOFAS026-08	S26	KU567259
Hypotrigona gribodoi	BOFAS022-08	S22	KU567260
Hypotrigona gribodoi	BOFAS241-15	4_17_1m	KU567261
Hypotrigona gribodoi	BOFAS062-08	S62	KU567262
Hypotrigona gribodoi	BOFAS016-08	S16	KU567264
Hypotrigona gribodoi	BOFAS004-08	S04	KU567265
Hypotrigona gribodoi	BOFAS030-08	S30	KU567268
Hypotrigona gribodoi	BOFAS029-08	S29	KU567269
Hypotrigona gribodoi	BOFAS021-08	S21	KU567270
Hypotrigona ruspolii	BOFAS135-15	2_26_3k2	KU567287
Hypotrigona ruspolii	BOFAS137-15	2_26_1k2	KU567276
Hypotrigona ruspolii	BOFAS192-15	2_6_6k2	KU567281
Hypotrigona ruspolii	BOFAS136-15	2_26_2k2	KU567283
Hypotrigona ruspolii	BOFAS193-15	2_6_5k2	KU567286
Hypotrigona ruspolii	BOFAS251-15	hr_4_kk	KU567285
Hypotrigona ruspolii	BOFAS250-15	hr_3_kk	KU567280
Hypotrigona ruspolii	BOFAS254-15	hr_7_kk	KU567288
Hypotrigona ruspolii	BOFAS253-15	hr_6_kk	KU567277
Hypotrigona ruspolii	BOFAS194-15	2_6_4k2	KU567282
Hypotrigona ruspolii	BOFAS256-15	hg_5_kk	KU567279
Hypotrigona ruspolii	BOFAS255-15	hg_4_kk	KU567284
Hypotrigona ruspolii	BOFAS252-15	hr_5_kk	KU567278
Hypotrigona ruspolii	BOFAS258-15	4_2K3	KU567294
Hypotrigona ruspolii	BOFAS259-15	5_2K3	KU567297
Hypotrigona ruspolii	BOFAS261-15	7_2K3	KU567295
Hypotrigona ruspolii	BOFAS266-15	14_2K3	KU567292
Meliponula lendiliana	BOFAS141-15	2_24_4K2	KU146611
Meliponula lendiliana	BOFAS146-15	2_22_4K2	KU146608