Discrimination of *Hypotrigona* species (Apidae: Meliponinae) in Kenya using nest architecture, cephalic secretions and molecular tools

by

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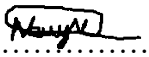
Declaration

I, Ndungu Nelly Njeri, declare that the thesis which I hereby submit for the degree of Doctor of Philosophy in Entomology at the University of Pretoria, is my own work and has not been previously submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:  ……..……..

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Dedication

I dedicate this thesis to my parents, Lucy Wairimu and the late Benjamin Ndungu Choroma.

To my dear husband, Joseph Mucugu; Children, Francis Ndungu, Patrick Waruiru, and Christine Wahu for moral and spiritual support.

Above all I thank God for divine favour.

Publications and Thesis organisation

Chapters in this thesis are presented in the form of publications and manuscripts as shown below. Hence, there are overlap of information and differences in the format of presentation.

Chapter 2. N.N. Ndungu, A.A. Yusuf, S.K. Raina, D.K. Masiga, C.W.W. Pirk & K. Nkoba (2018) Nest architecture as a tool for species discrimination of *Hypotrigona* species (Hymenoptera: Apidae: Meliponini). In press - *African Entomology*

Chapter 3. Ndungu, N. N., Kiatoko N., Sole C.L., Pirk, C. W. W., Yusuf A. A., Raina, S. K. and Masiga, D. K. (2018) Resolving taxonomic ambiguity and cryptic speciation of *Hypotrigona* species through Morphometrics and DNA barcoding. *Journal of Apicultural Research*, 57:354-363. DOI: 10.1080/00218839.2018.1426348

Chapter 4. Ndungu, N. N. Kiatoko N., Masiga, D. K. Raina S. K, Pirk C. W. W. and Yusuf A. A. (2018) Compounds extracted from heads of African stingless bees (*Hypotrigona* species) as a prospective taxonomic tool. *Chemoecology*, 28 (2):51-60, DOI:10.1007/s00049-018-0256-6

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Discrimination of *Hypotrigona* species (Apidae: Meliponinae) in Kenya using nest architecture, cephalic secretions and molecular tools

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Abstract

Stingless bees are important pollinators contributing significantly to biodiversity and food security. Stingless bees produce honey that has high medicinal value that fetches higher prices compared to the honey produced by honey bees (*Apis mellifera*). However, identification and classification of Afrotropical stingless bees, which is key and important for their domestication, still remains ambiguous and solely reliant on the use of morphological features. In this study, an integrative taxonomy approach was applied to identify and differentiate three *Hypotrigona* species: *Hypotrigona gribodoi*, *H. ruspolii* and *H. araujoi* that are found in Kenya. Nesting sites, nest architecture, morphometrics, DNA barcoding and chemotaxonomy using whole head extracts were employed as complementary tools to identify and differentiate the *Hypotrigona* species.

Colonies of the three *Hypotrigona* species from Kakamega forest and Mwingi, Kenya were nested at meliponary on the campus of *icipe* in Nairobi, Kenya. Nest sites, nest entrance (colour and sizes) and nest architecture (brood cells arrangement and sizes, honey and pollen pots sizes, presence or absence of involucrum and colour of propolis) were recorded. It was found that nest sites are specific with *Hypotrigona gribodoi* nesting mostly in crevices of mud walls in homesteads, while *H. ruspolii* and *H. araujoi* nest in specific indigenous tree species found in Kakamega forest. The colour of external nest entrances varies between species. Those in *H. araujoi* were yellowish-brown; white or cream in *H. gribodoi* while *H. ruspolii'*s were dark brown. There is an internal nest entrance in *H. gribodoi*, which is absent in the other species. Brood cells

are clustered in *H. gribodoi's* and *H. ruspolii's* nests, whereas *H. araujoi'*s form vertical semicomb-like layers. The surface area of the apical opening of the entrance tube and volumes of brood cells, honey and pollen pots differ significantly between the three *Hypotrigona* species. Using veins on the right forewing and hind leg for morphometrics analysis, *H. gribodoi* and *H. ruspolii* were separated from *H. araujoi*. However, there is an overlap between *H. gribodoi* and *H. ruspolii*. On the other hand, using mitochondrial DNA, *COI* gene, the three *Hypotrigona* species were clearly separated. A lower genetic distance exists between *H. araujoi* and *H. gribodoi* from Kakamega (1.4%) than between *H. gribodoi* collected from Kakamega and *H. gribodoi* from Mwingi (4.3%). Using gas chromatography and mass spectrometry, analysis of extracts from the head of workers, 50 components belonging to six chemical classes; hydrocarbons, aldehydes, alcohols, terpenoids, steroids and fatty acids were identified. Twenty-nine compounds were found in the cephalic extracts of *H. araujoi*, 26 in *H. gribodoi* and 33 in *H. ruspolii*. Workers were successfully grouped into their respective species and colonies using sixteen components among which: heptacosene, heptacosanol and octadecanol contributed most to the separation into species.

In conclusion, nest entrance and nest architecture show variation between the three *Hypotrigona* species. Use of morphometrics and molecular taxonomic approaches (DNA barcoding) provides a convenient, robust and reliable way to identify *Hypotrigona* species. The large genetic distance between *H. gribodoi* collected from Kakamega and Mwingi suggests that *H. gribodoi* ex-Mwingi may be a new undescribed putative species. Gas chromatographic and mass spectrometric analysis of head extract reveals differences in secretions among the *Hypotrigona* species. Thus, integrative taxonomy tools applied in this study provides a valuable alternative to identify *Hypotrigona* species. In addition, this study indicates the need for a thorough revision of *Hypotrigona* species.

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CHAPTER 1

General Introduction

Stingless bees (Apidae: Meliponini) are important in the pollination of plants (Slaa et al. 2006; Heard 1999; Kiatoko et al. 2014). Foragers will visit one plant species at a time, and thereafter recruit others by providing information on the available floral resources (Kremen et al. 2002; Albano et al. 2009; Potts et al. 2010; Slaa et al. 2006). In addition, stingless bees produce honey, which has high medicinal and economic value as compared to that from honey bees (Vit et al. 2004). Proceeds from the honey sales serve as a source of income, and its prices are higher in comparison to those from honey bees (Kiatoko et al. 2016). Unlike honey bees (Apini), stingless bees (Meliponini) have various advantages: they do not sting and thus not harmful to human and domestic animals, and are able to forage effectively in greenhouses (Slaa et al. 2006). Thus, stingless bees are important alternative and/or complement to honey bee pollination due to the colony losses that is currently affecting managed honey bee colonies worldwide (Cortopassi-Laurino et al. 2006). However, there are a few challenges in stingless beekeeping (meliponiculture). These include; insufficient domestication and meliponiculture technology, poor knowledge of pollination and commercial production, and poor taxonomic knowledge (Pinheiro-Machado 2002; Eardley 2004; Cortopassi-Laurino et al. 2006).

According to Eardley (2004), 19 species of stingless bees in six genera have been described in Africa; *Meliponula* Cockerell, *Dactlyrulina* Cockerel, *Liotrigona* Moure, *Plebeina* Moure, *Hypotrigona* Cockerell and *Cleptotrigona* Moure. In Kenya, stingless bee species are widely distributed especially in warm naturally forested areas such as Kakamega and Mwingi (Macharia et al. 2007; Eardley 2004). The species present in Kakamega include *Meliponula bocandei, M. (Trigona) ferruginea, M. lendliana, Plebeina hildebrandti, Hypotrigona ruspolii, H. araujoi, H. gribodoi* and *M. (Meliplebeina) becarii*. Whilst, stingless bee species found in Mwingi are *H. gribodoi* and *M. (Trigona) ferruginea* (Raina et al. 2011; Nkoba et al. 2012; Ndungu et al. 2017).

While five of the six genera of stingless bees found in Kenya have been identified using both morphometrics and mitochondrial DNA (Ndungu et al. 2017), this has not been done for *Hypotrigona*. Although studies on *Hypotrigona* have been neglected, they have the potential of being reared and are important pollinators (Kiatoko et al. 2014).

There is an increased demand for pollination, especially for horticulture crops (Slaa et al. 2006) and as such, there is an urgent need to find alternative pollinators to complement honey bees. Thus, there is a need to conserve the *Hypotrigona* species. However, their existence is threatened by forest destruction leading to loss in their nest sites. To conserve and domesticate *Hypotrigona* species, sound knowledge of their taxonomy is imperative. However, the challenge is that *Hypotrigona* species are difficult to identify and differentiate (Eardley 2004). Attempts have been made in past to distinguish the following three *Hypotrigona* species; *H. araujoi*, *H. gribodoi* and *H. ruspolii*. For instance, Guiglia (1955) described *H. gribodoi* morphologically, while Michener (1959) carried out a breeding experiment to prove that *H. araujoi* and *H. gribodoi* do not mate and are indeed biological species. Moure (1961) separated *H. gribodoi* and *H. araujoi* based on the ratio of the whole body lengths. Whilst Eardley (2004) generated taxonomic keys using morphologies of the worker's legs, wings, head and thorax, in which he showed that the character differentiating *H. ruspolii* from the other two species is an imaginary line posterior to its midline, which in *H. gribodoi* and *H. araujoi* is in the middle of the hind tibia.

Despite these attempts to differentiate *Hypotrigona* species, it remains difficult to identify these species without the relevant taxonomic expertise needed to interpret available taxonomic keys. There is therefore a dire need for tools that could be used in differentiating the three *Hypotrigona* species that can be applied both on a large scale and at varying levels of taxonomic expertise. In the present study, an integrative taxonomy was applied to identify and differentiate *Hypotrigona* species. These involved the use of nest site, nest architecture, morphometrics, mitochondrial DNA sequences and chemical profiling of head extracts.

Classification of stingless bees

Stingless bees belong to the Phylum, *Arthropoda*; Class, *Insecta*; Order, *Hymenoptera*; Suborder, *Apocrita*; Superfamily, *Apoidea*; Family, *Apidae*; Sub family, *Apinae* and Tribe, *Meliponini* (Michener 2007). They are widely spread, compared to honey bees (Apini), and are native to most of the tropical and equatorial regions of the world (Ruttner 1988). The total number of species within the Meliponini is still controversial but is estimated to be over 500 (Camargo and Pedro 1992; Rasmussen and Camargo 2008; Michener 2007; Eardley 2004). Most of the 500 species of stingless bees in the pantropical regions of the world have not been studied in depth. Consequently, the taxonomy of much of this group is still fundamentally uncertain (Carmargo et al. 1988; Michener 2007). The taxonomy of stingless bees is sometimes uncertain, where different taxonomists have different opinions on nomenclature and thus species names change overtime (Wille 1983; Roubik, 1992; Michener, 2007; Eardley 2004). For the African stingless bees, 19 species from the genera *Meliponula* Cockerell, *Dactylurina* Cockerel, *Plebeina* Moure, *Liotrigona* Moure, *Hypotrigona* Cockerell and *Cleptotrigona* Moure have been described. (Eardley 2004). A study on the following four Meliponini genera found in Kenya: *Meliponula, Dactylurina, Lendliana* and *Plebeina* were recently conducted (Ndungu et al. 2017). However, no such studies have been carried out on the species found in the *Hypotrigona* genera despite their importance as pollinators.

Biology of stingless bees

There are two eusocial bee tribes in the afro tropics; honey bees, Apini and stingless bees, Meliponini (Michener 2007). In this section, comparisons are made based on these two tribes. Meliponini and Apini are similar in that they have morphologically distinct workers, queens and drones (Peters et al. 1998; Michener 1974). However, the Meliponini differ from Apini in many morphological and biological ways including; the possession of fewer wing veins, a non-functioning sting and a penicillum (a brush of long setae on the outer apical surface of the hind tibia) (Michener 1990, 2007). In contrast to Apini, the species included in the tribe Meliponini show considerable

variation in size, nesting sites and nest architectures (Michener 1974). According to Costa et al. 2003, the key attribute that discriminates meliponini from other corbulate bees is the lack of an auricle on the hind basitarsus. The meliponini generally mate only once (monoandry) and do not use pure wax to build the brood cells, but mix it with plant resin (Roubik 2006). They do not freely swarm to form new colonies but make new nest where old workers feed the new queen for some time before the colony stabilises (Roubik 2006). Unlike Apini and bumble bees, meliponini colonies show reduced dispersal (a few hundred meters at the most) (Engels and Imperatriz-Fonseca 1990) and this hinders wide spreading of their mitochondrial genes (Quezada-Euán et al. 2007). Meliponini queens are produced in larger numbers and if an actively reproducing queen is present in the nest it kills the newly hatched queen (Peters et al. 1999). Meliponini bees also produce brood like solitary bees, but raise them through mass provisioning, where food is placed in the brood cell and an egg laid on the food. Thereafter, the cell is sealed for the egg to develop to adult. This is in contrast to the apini who feed their developing larvae progressively as it develops into adult (Roubik 2006).

Importance of stingless bees

Stingless bees are small in size, thus visit small flowering plants that provides small amounts of pollen and nectar (Heard 1999) in comparison to plants pollinated by other bees. Stingless bees pollinate food crops, for instance, *H. gribodoi* has been shown to be an effective pollinator of capsicum, improving its yield in terms of fruit set, weight, number of seeds and the size of the fruit (Kiatoko et al. 2014). Unlike honey bees, stingless bees produce small quantities of honey, which is difficult to extract hygienically. However, research has shown that honey from stingless bees has more pharmacological values compared to that from honey bees (Torres et al. 2004; Garedew 2003; Vit et al. 2004). Stingless bee honey production has been realised in several regions of Africa (Kwapong et al. 2010), Central America and Australia (Vit et al. 1993). Currently there are commercial production of stingless bee honey in Kenya (Kiatoko et al. 2016) and Ghana (Kwapwong et al. 2010). Propolis from stingless bees has been shown to have antibacterial, antifungal and anti-inflammatory properties, thus making it important in medical care (Sawaya et al. 2009; Muli et al. 2008).

Identification of Stingless bees

Eardley (2004) has extensively described the morphological features of the workers of African stingless bees. However, there is still a challenge in the use of morphological features for the species which are morphologically similar (See Appendix IV). Apart from use of morphological features, other features that can be used to identify them include nest sites and nest architecture (Michener 1974; Roubik 2006). Indeed, the nest sites of stingless bees are specific (Wille and Michener 1973; Michener 1974; Hubbell and Johnson 1977; Roubik 2006; Kajobe 2007; Kajobe and Roubik 2006; Eltz et al. 2002; Nkoba et al. 2012). For instance, *H. gribodoi* and *M. ferruginea "*reddish brown" prefer nesting in crevices found on walls of mud houses (although some *H. gribodoi* are found nesting on trees). *Meliponula bocandei* and *M. ferruginea* "black" prefer cavities in forest trees, while *M. lendliana* nests in underground cavities (Kajobe 2007; Nkoba et al. 2017; Roubik 2006). The brood cell arrangements in nests of stingless bees are also species specific with combs arranged vertically or in clusters depending on the species (Wille 1983; Roubik 2006).

Apart from using morphology, nest sites and nest architecture (Roubik 2006), identification of African stingless bees has recently been carried out using morphometrics (Ndungu et al. 2017). Morphometric analysis techniques have been undertaken using various software packages that consider the morphometric outlines of individuals, and thus it can be applied to differentiate inter- and intraspecific groups of offspring of different queens in the same colony (Carvalho et al. 2011). 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 high level of expertise.

The wings venation of stingless bees are greatly reduced with the marginal cells opening apically and the distal parts of the veins much narrower than the basal parts that are located near the stigma (Michener 2007). The stigma is large but the pre-stigma is almost absent. First and second sub marginal cells are often unrecognisable and the third

cell is not defined (Michener 2007). Field experiments show that morphometrics can reveal a high degree of polymorphism among specimen (Dujardin et al. 1997). Thus, in a recent morphometric study, veins of the radial and cubital cells were selected as important characters that could separate species (Ndungu et al. 2017). Wing morphometrics has been applied in the separation of *Tetragonula iridipennis* into two clusters (Francoy et al. 2016) to distinguish five species of stingless bees in Brazil and discriminate four stingless bee species found in Ghana (Combey et al. 2013). Hence, it is recommended as a useful tool for studies in biodiversity and conservation programs (Francoy et al. 2009).

On the other hand, molecular sequence data have become more available to study taxonomy, population genetics, systematics and evolutionary trends in bees (Brito et al. 2013; Franck et al. 2004; Magnacca and Brown 2010; May-Itzá et al. 2012. This involves the use of mitochondrial DNA (mtDNA) that is often used for species and subspecies characterisation, phylogenetics and systematics (Weinlich et al. 2004) because it is maternally inherited, do not recombine, has high-mutation rates and its small molecular size (Barni et al. 2007).

Application of DNA barcoding in taxonomy

The 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). This integration of morphometrics and DNA barcoding have been applied successfully to separate what was previously known as *Melipona yucatana* from Mexico and Guatemala, into two distinct species (May-Itzá et al. 2010) and in the separation of two morphospecies, *Meliponula ferruginea* “reddish brown” and “black” (Ndungu et al. 2017). In addition, using morphometrics and molecular tools like Internally Transcribed Spacer (ITS) of ribosomal gene showed that *Melipona bechii* from southern Mexico and Central America are different (May-Itzá et al. 2012). Similar approach of combining morphometrics and mtDNA analysis was used to regroup the 24 subspecies of *A. mellifera* into four evolutionary lineages (A, C, M and O) (Barni et al. 2007).

DNA barcoding involves the application of sequence diversity in short standardised gene segments. In the animal kingdom the frequently used barcode gene is the Cytochrome-C-oxidase I (*COI*) gene that is composed of approximately 648bp (Hebert et al. 2004). DNA barcoding has received much attention due to its ability to identify species including cryptic species (Khamis et al. 2012; Hebert et al. 2004; Hurtado-Burillo et al. 2013). It is applied in less studied taxonomic groups to sort specimens to genus level. Current results show that this record will be very effective in the more than 955 species of animals that possess distinctive *COI* sequences (Hebert et al. 2003; Hajibabaei et al. 2007). The mitochondrial markers are haploid and unipolarly inherited, and can be applied in population level studies.

Normally, *COI* sequences differ between species of insects by 3-10% (Hebert et al. 2003; Monaghan et al. 2006). However, in some sibling species lower sequence variation of 0.32% has been observed (Hebert et al. 2004). Molecular barcoding only assists in confirming species identification and therefore ecology, morphology and behaviour cannot be ignored in taxonomic studies. Molecular barcoding has the capability to facilitate identification of any life-history stage, sex, and tissue of a properly curated sample of the species in query. DNA barcoding alone has shown possibility that cryptic species of *Scaptotrigona mexicana* may exist, and thus the technique can discriminate stingless bee species in collaboration with the existing taxonomic descriptions (Hurtado-Burillo et al. 2013). Recently DNA barcoding has been used to separate five stingless bees found in Kenya and to detect cryptic species in *Meliponula ferruginea* “morphospecies” (Ndungu et al. 2017).

Although DNA barcoding has been applied widely for species identification and discovery, it is not without critique. Some of these criticisms include the standardisation in DNA barcoding, where one or more of the reference genes used is still debatable because it may not fit all organisms (Mortiz and Cisero 2004). Taylor and Harris (2012) had criticised DNA barcoding since it does not apply next generation sequencing and recommended that this should be incorporated to enhance high throughput. This has been achieved recently through pollen DNA metabarcoding to study plants (Sickel et al. 2015; Bell et al. 2016, 2017). Pseudogenes (mitochondrial DNA transferred to nuclear

genome) have also been reported to hinder success in barcoding, however, in studies where morphological and ecological and COI data are combined, then it does not pose a problem (Leite 2012). Lastly, DNA barcoding gap has been raised as a concern, even though the 3% difference threshold has been indicated as sufficient genetic distance to separate different species (Meyer and Paulay 2005).

Cephalic chemical profiles of stingless bees and its application in taxonomy

Stingless bees have complex communication systems that are regulated by secretions of both mandibular and labial glands (Engels et al. 1990). In a recent review, Leonhardt (2017) shows that the composition of cephalic volatiles of Neotropical stingless bees are species and nest specific, and that taxonomic relations can be deduced from similarity in patterns of these volatiles. In addition, interspecific competition could induce genetically based variation in volatile signals to enable workers to discriminate between nest mates and alien conspecifics (Francke et al. 2000). Gracioli-Vitti et al. (2012) indicated that stingless bees produce species–specific chemicals that play a role in communication, defence and mating. Apart from secreting pheromones or kairomones that induce defensive or aggressive behaviour just like any other eusocial insects, stingless bees are the only ones known to secrete trail pheromones used to recruit foragers to food sources (Kerr et al. 1963; Schorkopf et al. 2009). It has been shown that cephalic extracts differ between castes (queen and workers) and sexes (drone and workers) (Francke et al. 2000). Further, cephalic extracts are different between newly emerged workers and foragers (Poiani et al. 2014). Previous studies on 11 Brazilian social stingless bee species from the genus *Tetragonisca* (Francke et al. 2000) and two *Frieseomelitta* species (López et al. 2002) show variations in their chemical profiles.

The rationale and motivation of the study

Available keys based on morphological features used in the identification of *Hypotrigona* species are difficult, ambiguous and requires taxonomic expertise. In most cases, these keys are outdated and not detailed enough to separate *Hypotirgona* species. This research seeks to solve the problem in the identification of stingless bees within *Hypotrigona* found in Kenya, by developing simple and easy integrative taxonomic

tools (which can be used by stingless bee farmers and researchers) which do not require taxonomic expertise to interpret.

The difficulties, ambiguities and reasons for resolving taxonomic issues are discussed in the General introduction (**Chapter 1).** Four subtopics have been summarised, the classification of stingless bees, their economic importance, biology and their identification. In the first objective, nest architecture and nest sites were applied to identify *Hypotrigona* species. It has been shown that nest architecture and nest sites can be used to identify stingless bee species especially in the field (Camargo and Wittmann 1989; Eardley and Kwapong 2013). This is addressed in **Chapter 2,** which gives the results of the nest architecture of three *Hypotrigona* species and how nests sites and nest architecture can be used to identify these species in the field. The second objective was to discriminate the three *Hypotrigona* species, using morphometrics and DNA barcoding. The usefulness of combining morphometrics and DNA barcoding is shown in **Chapter 3** by identifying the different *Hypotrigona* species. The third objective was the use of chemical analysis of the cephalic volatiles from the heads of workers to discriminate between *Hypotrigona* species. The compositions of cephalic volatiles in Neotropical stingless bees are specific and similarities in patterns indicate taxonomic relations (Francke et al. 2000). Despite many studies on the labial and mandibular pheromones of the Neotropical stingless bees, no such analysis has been carried out on the stingless bees in Kenya, specifically those from the genera *Hypotrigona*. In addition, most studies analyse extracts directly, thus non-volatile components like fatty acids that are known pheromones in other bees are often missed or under reported. **Chapter 4** presents the compositions of chemical components from whole head extracts and how these can be used to identify three *Hypotrigona* species found in Kenya. The study gives a detailed description of nest sites, nest architecture, morphometrics, mitochondrial DNA data and chemical profile of the head extracts, which are all lacking in the current literature. This research contributes to the current knowledge of the African stingless bees, specifically *Hypotrigona* that is currently difficult to identify. All the results are summarised and brought together in **Chapter 5** as a synthesis that includes the general conclusions from the study and recommendations for future studies.

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CHAPTER 2

Nest architecture as a tool for species discrimination in *Hypotrigona* species (Hymenoptera: Apidae: Meliponini)

Abstract

Hypotrigona species are difficult to identify morphologically. Here, we show that nest sites and nest architecture can be used to identify three *Hypotrigona* species found in Kenya. *Hypotrigona gribodoi*, *H. araujoi* and *H. ruspolii* colonies from Kakamega forest and *H. gribodoi* from Mwingi, were collected and placed in a meliponiary at the International Centre for insect Physiology and Ecology (ICIPE). The following parameters were recorded: nest sites, internal nest entrance, external nest entrances colour and size, nest sizes (volume of brood cells, honey pots and pollen pots), brood cells arrangement and the presence or absence of involucrum (cerumen covering brood). It was found that nest sites are species specific. *H. gribodoi* nests mostly in crevices in mud walls, while *H. ruspolii* and *H. araujoi* nest in specific tree species in indigenous forest. The colour of external nest entrances varies between the species. In *H. araujoi* they are yellowish- brown, white or cream in *H. gribodoi* and dark brown in *H. ruspolii*. There is an internal nest entrance in *H. gribodoi,* which is absent in the other two *Hypotrigona* species. Brood cells are clustered in *H. gribodoi* and *H. ruspolii* whereas in *H. araujoi'*s they form vertical semicomb-like layers. The area of the apical opening of the entrance tube and volumes of brood cells, honey and pollen pots differ significantly between the three species. Therefore, nest sites and nest architecture can be used to identify three *Hypotrigona* species

Key words: Comb structure, stingless bees, nest entrance, *H. gribodoi, H. araujoi, H. ruspolii*

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Introduction

Stingless bees are found in the tropical regions of the world (Michener 2000; Michener and Grimaldi 1988; Rasmussen et al. 2010) where they play a vital ecological role in the pollination of many plant species (Heard 1999; Nkoba et al. 2014; Slaa et al. 2006). Although stingless bees produce less honey than honey bees do, their honey is important for subsistence in many rural communities (Eardley and Kwapong 2013; Nkoba et al. 2012, 2016). Unlike honey bees for which only 11 species have been described in the single genus *Apis* (Michener 2000); stingless bees taxa are diverse with over 60 genera in which over 600 species have been reported so far (Michener 2000; Rasmussen and Cameron 2010). Contrary to Neotropical stingless bees where several studies have been carried out on their taxonomy, biology, ecology and genetics, African species have been less studied with their classification still vague (Eardley 2004; Eardley and Kwapong 2013; Michener 2000). The taxonomic revision by Eardley (2004) provides identification keys for all African stingless bees known at the time, based on their morphology. Six genera; *Meliponula* Cockerell, *Dactylurina* Cockerell, *Plebeina* Moure, *Hypotrigona* Cockerell, *Cleptotrigona* and *Liotrigona* Moure (Sakagami et al. 1993) have been identified that comprise 20 species (Eardley 2004) with 12 present in Kenya (Ndungu et al. 2017; Nkoba et al. 2012). *Hypotrigona* consists of four species namely, *H. ruspolii* (Magretti 1898), *H. gribodoi* (Magretti 1884), *H. araujoi* (Michener 1959) and *H. squamuligera* (Benoist 1973) (described as *H. penna* by Eardley 2004). *Hypotrigona squamuligera* occurs only in West Africa, while the three other species are present in different habitats in East Africa. *Hypotrigona* species are difficult for even taxonomic experts to differentiate due to their highly similar morphology (Eardley 2004; Michener 1990, 2000). However, the three *Hypotrigona* species were separated using morphometrics and molecular tools, (Ndungu et al. 2018a, see chapter 3) and chemical extracts from whole heads (Ndungu et al 2018b, see chapter 4). However, the molecular methods and chemical extracts are only feasible in the laboratory and hence cannot be used out in the field or by stingless bee keepers to enhance their domestication and conservation. The accurate identification of species is required for colony propagation that involves techniques such as queen production and colony division (Slaa et al. 2006).

Therefore, there is a need to develop easier ways of identifying *Hypotrigona* species in the field and in meliponiaries based on external and internal features of their nests.

Apart from using body morphology as a tool for species differentiation, nest architecture and nesting ecology are used to identify stingless bee species. The nest architecture of stingless bees in South America and Australia have been studied, however, little has been reported in Africa on nest architecture especially those of *Hypotrigona* species. Only Portugal-Araujo and Kerr (1959) and Michener (1959) reported some differences between nests of *H. gribodoi* and *H. araujoi.* A more comprehensive description of the nest architecture features for in-field identification of *Hypotrigona* species using the least destructive techniques is desirable.

In this study, detailed examination of the nest architecture was carried out in order to develop field identification tools for the three *Hypotrigona* species found in Kenya.

Materials and methods

Nest sampling and species identification

During 2014-2015, nests of *H. gribodoi, H. araujoi* and *H. ruspolii* were collected from two ecological zones in Kenya, namely Kakamega (0°09'N 34°50'E) a forest and Mwingi (0°51′S 38°22′E) a semiarid area (Fig. 2.1). Random searches for *Hypotrigona* nests were carried out in three habitats (forest, grasslands and homesteads) by looking for protruding nest entrances or foragers flying in and out of the nests (Kajobe 2007; Nkoba et al. 2012, 2017; Kwapong et al. 2010). *Hypotrigona araujoi* and *H. ruspolii* nests were mostly located in living tree species like *Croton silvaticus, Prunus africana, Funtumia africana, Antiaris africana* and *Olea capensis* within in the Kakamega indigenous forest. On the other hand, *H. ruspolii* nests mostly on *Cordia africana, Croton silvaticus, Prunus africana, Funtumia africana, Olea capensis* and *Ficus umbellate* within Kakamega forest. *Hypotrigona gribodoi* nests were found in crevices of mud house walls within homesteads surrounding the forest but not in the forest. Whilst in Mwingi, *H. gribodoi* nests were found in homesteads. The *Hypotrigona* specimens collected in the field were identified using the taxonomic keys developed by Eardley (2004) and Michener (1959). To separate the *Hypotrigona* species molecular

tools were applied (Ndungu et al. 2018, see chapter 3); this information was then used to relate to their nest sites and architecture.

In order to study the nest architecture of the three *Hypotrigona* species, colonies were collected from the wild (Kakamega and Mwingi) and for each colony the brood cells with queen and workers were transferred into an ICIPE-1H hive design (27 (l) × 8.5 (w) × 7.5 (h)) cm (Kiatoko 2012). In total 55 colonies were harvested. Thirty *H. gribodoi* colonies (15 *H. gribodoi* from Mwingi and 15 from Kakamega); 15 *H. ruspolii* from Kakamega and 10 *H. araujoi* from Kakamega. These colonies were settled at first in meliponiaries in Kakamega and Mwingi for three weeks, and later transferred to a meliponiary at ICIPE Duduville campus (1°13'S, 36°53'E) in Nairobi, Kenya.

Data collection and analysis

Nest entrance architecture

To assess the differences in nest architecture between the three *Hypotrigona* species, we measured the shapes, and determined the colour and surface area of the apexes of the nest entrances (Kiatoko 2012). We also recorded the presence or absence of an internal nest entrance structure. An internal nest entrance is defined as an extended external nest entrance tube that leads into the nest. A digital Vernier calliper (Gimbel Mexicana, S.A. DE C.V, Mexico) was used to take measurements of the minor (the shorter diameter) (R_1) and major axes (the longer diameter) (R_2) of the entrance tube. The shape of the apical opening of the entrance tube was determined by calculating the ratio R_2/R_1. A ratio equal to 1 ($R_1 = R_2$) was described as circular whilst those with ratios > 1 ($R_1 \neq R_2$) were described as oval. The cross sectional area (mm^2) of the entrance tube was calculated using the geometric formula for each of the shapes recorded. The surface area of a circular was calculated using the formula, $S = \pi R^2$ and for an oval opening, $S = \pi \times (R1) \times (R2)$ (Couvillon et al. 2008). All photographs were taken using a Nikon Camera Model l830, 34 × wide ED UR, optical 20 cm, 4.0 - 13.6 mm (Nikon Cooporation, Japan). The colours of the entrances were recorded with reference to RGB colour system (http://www.rapidtables.com/web/color/RGB_Color.htm#rgb).

Brood cell (arrangement, colour and sizes) and external pillars

Brood cells arrangement, colour and volume were recorded and photographed (Oldroyd and Pratt 2015; Roubik 2006; Roubik 1983; Michener 1959). Arrangement of brood cells were recorded as either, comb, semi-comb, spiral or cluster (Oldroyd and Pratt 2015). The presence or absence of outer pillars in the nests were also observed and photographed (Barbosa et al. 2013; Oldroyd and Pratt 2015; Roubik 2006). The sizes of the brood cells in terms of their volume were calculated for 30 brood cells collected from three hives per species. In total 90 brood cells were measured per species and the volumes calculated using the formula of a spheroid, $4/3\ \pi\ r^3$. The diameter and radius of brood cells were measured under a Zeiss microscope (Oberkochen, Germany) equipped with ZEN 2012 imaging software (version 1.1.2.0, Carl Zeiss Microscopy, GmbH) at a magnification of × 0.54.

Honey and pollen pots

The volumes of honey and pollen pots (mm^3) were calculated and the colour determined with reference to the RGB colour system (http://www.rapidtables.com/web/color/RGB_Color.htm#rgb-format). At least 15 honey and pollen pots were measured in three colonies of each species (*H. ruspolii* = 67, *H. araujoi* = 34 and *H. gribodoi* = 86). For pollen pots, *H. ruspolii* = 33, *H. araujoi* = 23 and *H. gribodoi* = 87. Volumes of the honey and pollen pots were calculated using the formulae of a spherical shape, $4/3\ \pi\ r^3$.

The involucra, colour of propolis and garbage dumping sites in the nest

The presence or absence of an inner involucrum and garbage sites in the nests were observed recorded and photographed (Barbosa et al. 2013; Oldroyd and Pratt 2015; Roubik 2006). The colour of the propolis was recorded with reference to RGB colour system (http://www.rapidtables.com/web/color/RGB_Color.htm#rgb-format).

Statistical analyses

The data on surface area for nest entrances and for the volumes of the brood cells honey and pollen pots were tested for normality and homogeneity of variance as assumed by analysis of variance (ANOVA). All these data did not significantly deviate from the normality assumption and homogeneity of variance. Hence, ANOVAs were performed to compare the three *Hypotrigona* species using the four parameters (surface area of the

apical opening of the entrance tube, brood cells volume, honey pots volume and pollen pots volume). Where ANOVA was significant, the mean for each parameter was separated using the Tukey HSD test. T-test was used to compare the surface area of the entrance, brood cells, pollen pots and volumes of honey pots for *H. gribodoi* from the two locations Mwingi and Kakamega to test for variation due to location. All statistical analyses were performed in R 3.2.3 (R Core Team 2015) with P values less than 0.05 being considered statistically significant.

Results

Nest entrance architecture

Most nests of *H. gribodoi*, *H. araujoi* and *H. ruspolii* had an external protruding entrance tube (Fig. 2.1a, d, g). Some *H. gribodoi* nests did not have a protruding entrance; instead they put soil, pebbles and resin at the entrance Fig. 2.1c). The shape, colour and area of the apical opening of the nest entrance varied among *Hypotrigona* species (Table 2.1). The colour of resin or sticky droplets scattered around the apex of the entrance tube varied between the three species as follows; yellowish white in *H. gribodoi* (Fig. 2.1a, b), reddish brown in *H. araujoi* (Fig. 1e, f) and dark brown in *H. ruspolii* (Fig. 2.1g, h). It was also observed that for nests of *H. gribodoi* and *H. araujoi*, the sticky droplets occurred at the outer surface where the entrance tube is attached to the substrate (Fig. 2.1i).

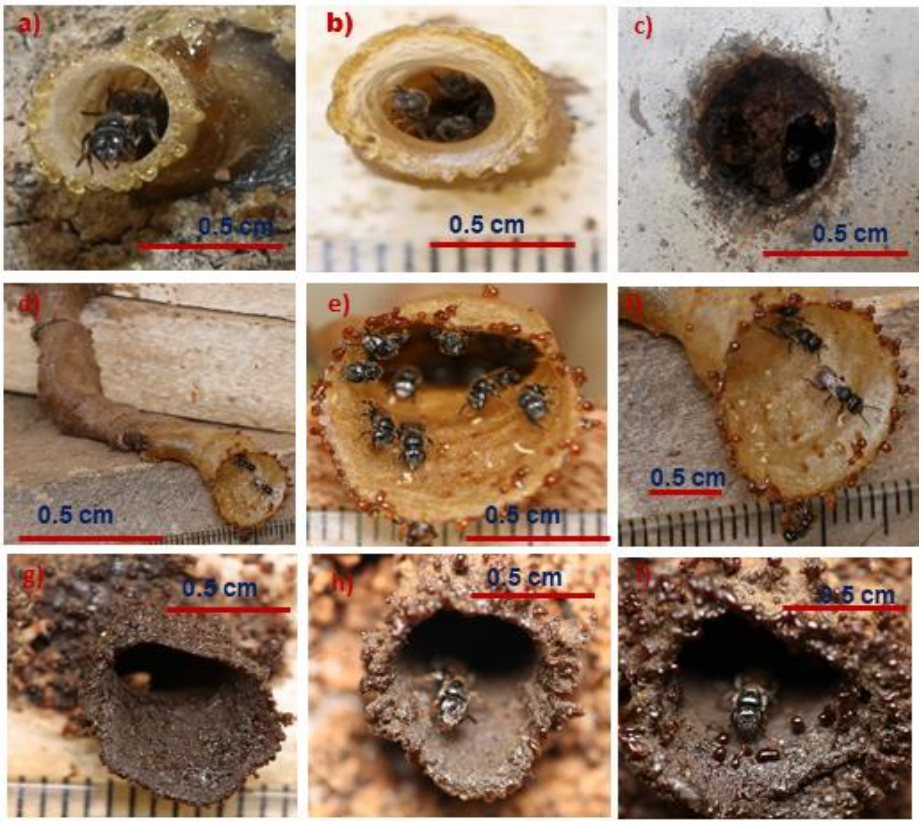

Fig. 2. 1. a-c. External nest entrances of *H. gribodoi*: **a**) circular, **b**) oval with resin droplets at the apex of the nest entrance. **c**) without protruding external nest entrance, soil pebbles and resin at the entrance. **d-f.**) External nest entrance of *H. araujoi*. **d**) Circular, also showing tube **e**) resin droplets at the apex of the nest entrance; **g-i**. Oval nest entrances **f**) *H. araujoi* **g)** *H.*

ruspolii; **h**) *H. gribodoi*; **i**) *H. ruspolii*, with resin droplets at the apex of nest entrance, without protruding tube.

The shapes of nest entrances varied between the three *Hypotrigona* species, ranging from circular or oval in *H. gribodoi* (Fig. 2.1a, b) and oval in both *H. araujoi* and *H ruspolii* (Fig. 2.1e-i).

The colour of the nest entrance tubes differed between the three *Hypotrigon*a species (Fig. 2.1). Nest entrance tubes were yellowish-brown in *H. araujoi*, white or cream in *H. gribodoi* and dark brown in *H ruspolii* (Fig. 2.1). The mean area of the apical opening varied significantly between the three species ($F_{2,38} = 86.33$, $P < 0.0001$) (Table 2. 1). There were significant differences between means of apical opening of the nest entrance of *H. gribodoi* (mean 8.42 ± 1.75 mm^2) and *H. araujoi* (128.00 mm^2 ± 15.08, Tukey HSD, $P < 0.0001$), and between *H. araujoi* and *H. ruspolii* (15.67 ± 2.48; Tukey HSD $P < 0.0001$). However, not between *H. ruspolii* and *H. gribodoi* (8.42 ± 1.75, Tukey HSD $P > 0.005$). The surface area at the apex of the nest entrance tube for *H. gribodoi* from Mwingi and from Kakamega differed significantly (T-test, $t_{39} = 8.57$, $P < 0.0001$).

Table 2. 1. Characteristics of nest entrances of the three *Hypotrigona* species

Characteristic	***Hypotrigona* species**		
	H. araujoi (N = 10)	***H. gribodoi (N = 15*, per location)**	***H. ruspolii (N = 15)***
Nest entrance shape	oval	circular, oval	oval
Nest entrance colour	yellowish-brown	white or cream	dark brown
Internal nest entrance	absent	Present	absent
Nest entrance apical opening area (mm^2).	128 ± 15.08 [c]	Kakamega = 8.42 ± 1.75[a], Mwingi = 12.2 ± 1.67 [d]	15.67 ± 2.48 [b]

Different letters in a row or column indicates significant differences. *N* = Number of samples

Structure of the internal nest differed among the species with those in *H. gribodoi* having an internal nest entrance tube, which led to the honey and pollen pots (Fig. 2.2a). Such were not observed in nests of *H. araujoi* and *H. ruspolii* (Fig. 2.2b, c).

Fig. 2. 2. a-c. Nest entrances in *Hypotrigona*. **a**) *Hypotrigona gribodoi*, internal nest entrance (red arrow), external entrance (black arrow); **b**) *H. araujoi* external nest entrance (black arrow) and **c**) *H. ruspolii* nest showing external entrance (black arrow).

Brood cells (arrangement, colour and sizes) and external pillars

The arrangements of brood cells colour and sizes varied between the three *Hypotrigona* species (Table 2. 2). In *H. araujoi*, brood cells are arranged spirally in vertical layers with cells in the same layer attach to each other forming a semicomb-like structure (Fig. 2.3a). Short pillars connected the different brood layers with the newest brood cells located on the outer most layers enclosing the older ones (Fig. 2.3a). One unique characteristic in the nests of *H. araujoi* were the presence of strong and long pillars attached on the top of the brood cells. These protruding pillars were longer (6 cm) and stronger than those between brood cell layers. Such external pillars where absent in nests of *H. gribodoi* and *H. ruspolii* (Fig. 2.3a).

The brood cells in *H. gribodoi* nests were arranged in clusters with short pillars connecting them and the newest cells were located above the older ones (Fig. 2.3b). Whilst in *H. gribodoi* brood cells were yellow-brown at pupal stage and yellow for newly capped cells. Similarly , brood cells in *H. ruspolii* nests were arranged in clusters with short, thin pillars connecting between some brood cells (Fig. 2.3c), and are located above the older brood cells. The newly capped cells were metallic cream with pale white pupal cells.

Fig. 2.3. a-c Brood cells arrangement in the *Hypotrigona* species. **a**) *H. araujoi* brood cells with old and new brood cells arranged to form semi-comb layers, new brood cells are on the outer layer. Strong pillars are also observed on the brood cells; **b**) Clustered brood cells arrangement as observed in *H. gribodoi*'s nest. The new brood cells are on top of old brood cells and **c**) Clustered arrangement of brood cells in *H. ruspolii* nest. New brood cells are constructed on top of the old ones.

Hypotrigona araujoi had the largest mean volume of brood cells, 12.7 ± 0.1 mm^3 while t he smallest volume was recorded in *H. ruspolii*, 6.07 ± 0.1 mm^3, and with *H. gribodoi's* being intermediate (Table 2.2). Mean volume of brood cells between the three *Hypotrig ona* species were significantly different ($F_{(2, 381)}$ = 807.4, P < 0.00001). Pairwise compar isons using Tukey HSD test show significant difference in the brood cell volumes betwe en *H. gribodoi and H. araujoi*; *H. ruspolii* and *H. araujoi* and *H. ruspolii* and *H. gribod oi* (p<0.0001). *H. araujoi* had the largest mean volume of brood cells (12.7 ± 0.12 mm^3) while the least volume was recorded for *H. ruspolii* (6.07 ± 0.09 mm^3), with *H. gribodoi* 's being intermediate (Table 2.2). Analyses using a T-test also showed that there were n o significant difference between the mean volume of brood cells of *H. gribodoi* collecte d from Mwingi and Kakamega ($t_{212.08}$=0.291, P = 0.771) (Table 2.2).

Table 2. 2. Brood cells arrangement, colour and volumes (mean±SE) of worker brood cells, in three *Hypotrigona* species

Characteristic	*Hypotrigona* species		
	H. araujoi (N = 90)	*H. gribodoi (N = 90,* per	*H. ruspolii (N =*

		location)	*90)*
Brood cells connecting pillars	absent	present	present
Brood cells arrangement	vertical semi- comb like layers	clustered	clustered
Colour of new brood cells	yellow	yellow	metallic cream
Colour of old brood cells	yellow brown	yellow brown	pale
Strong pillars on top of brood cells	present	absent	absent
Brood cell Volume (mm^3)	12.7 ± 0.12^{c}	Kakamega = 9.8 ± 0.13^{a} Mwingi = 9.7 ± 0.12^{a}	6.07 ± 0.09^{b}

*$P < 0.05$, ANOVA; Different letters in a row or column show significant difference. N = Number of samples

Honey and pollen pots sizes

The honey and pollen pots were mostly clustered in all three *Hypotrigona* species (Fig. 2.4a, b, c).

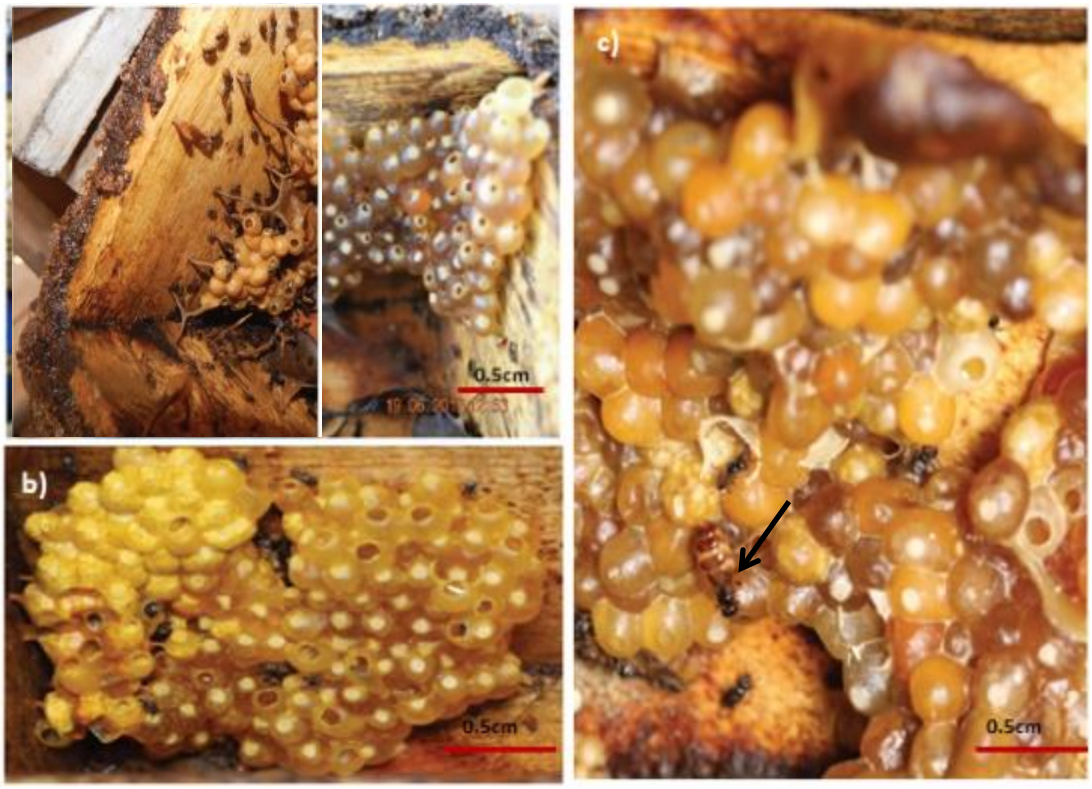

Fig. 2.4. Honey and pollen pots in; **a**) *H. araujoi* ***b***) *H. gribodoi* and **c**) *H. ruspolii.* The black arrow points to the queen.

Honey and pollen pots were spherical in shape. The mean volume of honey and pollen pots varied significantly among the three *Hypotrigona* species ($F_{2, 134} = 128.9$, $P < 0.0001$) and ($F_{2, 107} = 42.58$, $P < 0.0001$) (Table 2.3). There was also significant

difference in the volumes of honey and pollen pots of *H. gribodoi* collected from Kakamega and those from Mwingi (t_{78}= 20.631, $P < 0.0001$; $t_{90} = 28.3$, $p < 0.0001$).

Table 2. 3. Mean lengths and widths (±SE) of honey and pollen pots in three *Hypotrigona* species

Characteristic	***Hypotrigona* species**		
	H. araujoi	***H. gribodoi***	***H. ruspolii***
External involucra	absent	absent	present
Colour of propolis	reddish brown	light brown	dark brown
Honey pots volume (mm^3)	(*N* = 41) 168.29 ± 7.2[c]	(*N* = 41) Kakamega = 151 ± 8.4[a] (*N* = 45)Mwingi = 129 ± 9.4[d]	(*N* = 67)60.5 ± 2.1 [b]
Pollen pots volume (mm^3)	(*N* =23) 171 ± 14.2[c]	(*N* = 50) Kakamega =115 ± 7.4 [a] (*N* = 37) Mwingi = 122 ± 6.4 [d]	(*N* = 33) 65.2 ± 4.8 [b]

*$P < 0.05$, ANOVA Different letters in a row or column show significant difference. *N* = Number of samples

The involucra and colour of propolis

In contrast to *H. araujoi* and *H. gribodoi*, one unique characteristic in *H. ruspolii'*s nest is the presence of a dark brown inner involucrum which covered the brood cells (Fig. 2.5a, b, c). The colour of propolis used to seal cracks in hives are specific with the propolis deposited on hive gaps reddish brown in *H. araujoi*, light brown in *H. gribodoi*, and dark brown in *H. ruspolii* (Fig. 2.5d, e, f).

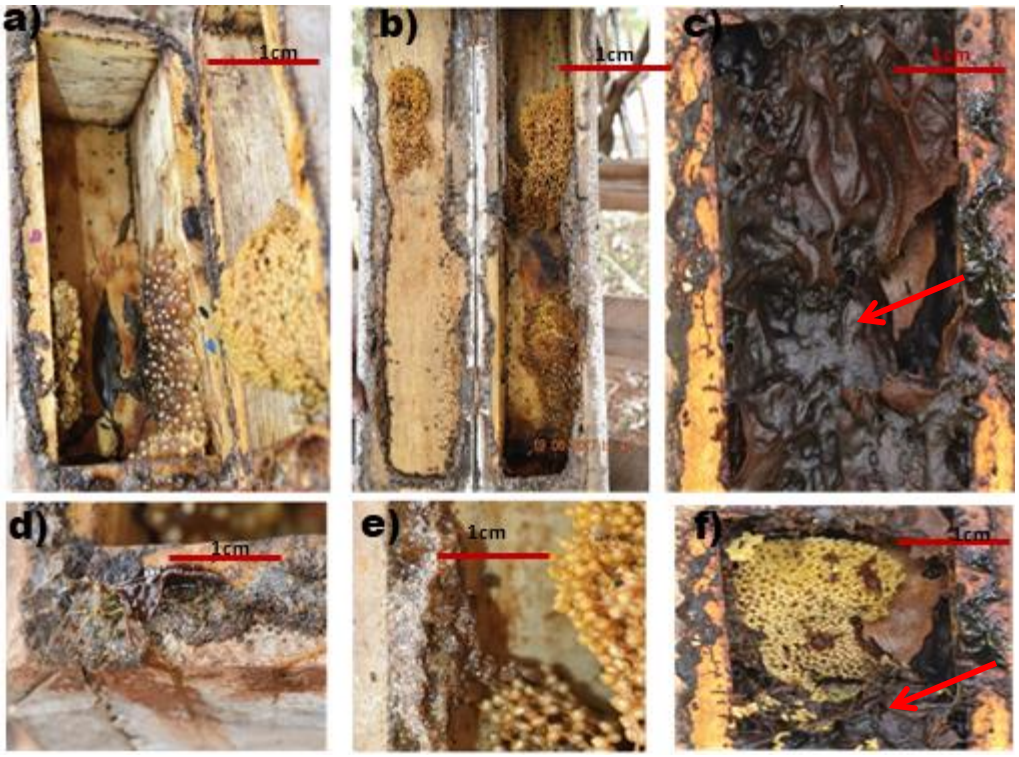

Fig. 2. 5a-c Overview of *Hypotrigona* nests **a**) *H. gribodoi*. **b**) *H. araujoi* **c**) *H. ruspolii*- Intact involucrum covering brood cells, honey and pollen pots (indicated by the red arrow). **d-f**) Propolis from *Hypotrigona* species. d) *H. araujoi*- reddish brown; e) *H. gribodoi*-light brown f) *H. ruspolii* -dark brown f) with the involucrum removed to show the brood cells underneath.

Garbage dumping sites

All three *Hypotrigona* species have a garbage site in their nests located near the honey and pollen pots and away from the brood cells. The garbage found was composed of dead honey bees, pieces of leaves, dead ants and other decomposing materials possibly discarded by workers and stuck on the inner flooring of the hive using sticky propolis. In *H. ruspolii* garbage sites are emptied by workers, whom transport garbage to the external entrance using their mandible and then dump it immediately outside the nest. Whilst in *H. gribodoi* and *H. araujoi*, workers remove garbage differently by flying away with the waste as they forage.

Discussion

The general nest architecture in *H. gribodoi*, *H. araujoi* and *H. ruspolii*, consists of four main compartments: an external nest entrance tube; old and new brood cells; honey pots,

pollen pots and a garbage-dumping site. In this study, key differences in nest architecture including the structure of nest entrances, arrangement, size and structure of brood cells, honey and pollen pots, colour of propolis and presence or absence of involucrum were described. These can be used as identification tools for the three species of *Hypotrigona*. According to Eardley (2004), *Hypotrigona* species are the most difficult to identify using morphological features. Stingless bees nest sites and nest architecture has been shown to be specific and can be used for species identification (Rasmussen and Camargo 2008; Roubik 2006). We observed that *Hypotrigona* species nest on different substrates in the different agro-ecological zones studied. In Kakamega and Mwingi, *H. gribodoi* nests in crevices in mud walls, dry tree logs and rocks. Whilst *H. araujoi* and *H. ruspolii* nest in pre-existing hollows in trunks and branches of trees found in the forest. Trees such as *Prunus africana* in which *H. ruspolii* and *H. araujoi* nested are known to be used in traditional medicine to inhibit lipid peroxidation and to treat symptoms associated with Benign Prostate Hyperplasia (BPH) (Hass et al. 1999). The dry climatic conditions in Mwingi, a semiarid area where the land has been degraded due to anthropogenic activities such as farming (Kaloi et al. 2005) could be a limiting factor for habitation of *H. araujoi* and *H. ruspolii*.

The colour of the external nest entrance tubes in nested colonies of *Hypotrigona* were white or cream in *H. gribodoi*, yellowish-brown in *H. araujoi* and dark brown in *H. ruspolii*. It was observed that though the nests were transferred into hives and later transferred from Kakamega and Mwingi to ICIPE away from the forest, the bees constructed nest entrances of the same colour as in their natural habitat. The colouration of the entrances appeared to be independent of environmental conditions as shown by the translocation of the nests. This was an indication that, inherent factors like mixing of wax and resin perform an important function in creating the specific colour of the entrance tubes. We suggest that the variation in colour in different species could be a result of the specific plant resins that each bee species forages upon for materials used in the construction of these nest entrances. Therefore, it is possible to use the colour of entrance to identify *Hypotrigona* species. In addition, the differences in nest entrances observed for the three *Hypotrigona* species provide a viable character that can be used for field identification. Nest entrances are smaller in *H. gribodoi* while those of *H.*

araujoi are wider among the species studied. Nest entrance architecture is important for the bees because it allows access for foragers and at the same time assist guard bees standing at the entrance to exclude intruders (Grüter et al. 2010). Narrow entrances are said to keep away intruders, while wider entrance favours foraging as it allows bees to leave and enter the hive easily (Biesmeijer et al. 2007; Roubik 2006). Only one or two guard bees are found at nest entrances in *H. gribodoi* and *H. ruspolii* making this an adaptive feature for this species in contrast to *H. araujoi*. In *H. araujoi*, nest entrances are wider that those of the other two species, and to better protect the nest a lot of resin need to be deposited around the tip and at the outer surface where the entrance tube is attached to the substrate with six to eight highly aggressive guards always present (personal observation). According to Roubik (2006), small size bees with wide entrance are shown to be highly defensive. Indeed, Michener (1959) showed that *H. araujoi* were more aggressive than *H. gribodoi*, which is in line with our findings on the size of the entrances.

A unique characteristic observed in *H. gribodoi* colonies was that, the outer entrance tube leads to an internal entrance tube that ends near the storage pots. This confirms previous observations by Bassindale (1955) on *H. gribodoi* nests in Ghana when he described internal entrances in this species. This indicates that internal nest entrance tubes could be used to lead the foragers to the storage pots while on the other hand disorienting non-nest mates and intruders

The worker brood cells in *H. gribodoi* and *H. ruspolii* form a cluster type arrangement which is a characteristic known to occur in primitive bees (Kerr and Maule 1964; Wille 1964). However, a specific characteristic was observed in *H araujoi* where brood cells are arranged in vertical layers of semicomb-like structures and thus could be more advanced in terms of their evolution compared to those in *H. gribodoi* and *H. ruspolii* (Ndungu et al. 2018). In addition, *H. araujoi* was the only species that had pillars emerging at the top of the brood mainly for attachment to the roof of the nest (Fig. 2.3).

None of the three species had inner involucra covering the brood cells, as is commonly present in most genera of African stingless bees (Barbosa et al. 2013). However, dark brown outer involucra covering the brood cells, honey and pollen pots were observed in nests of *H. ruspolii*. The construction of involucra has been shown to be an adaptation to

maintain optimum temperatures for growth of developing larvae in the brood cells and may also be relevant for humidity and defence against predators, parasites and pathogens (Barbosa et al. 2013; Figueiredo-Mecca et al. 2013; Rasmussen and Camargo 2008). The presence of external involucra is a specific characteristic that can be used to identify *H. ruspolii* in the field or when dealing with hives in the meliponiaries. An involucrum either surrounds the brood cells (brood cells involucra), or it surrounds both brood cells and storage pots (external involucra). Involucra are characteristics of nests in primitive stingless bee species (Rasmussen and Camargo 2008). Therefore, based on the occurrence of an involucrum and studies of mitochondrial DNA sequences (Ndungu et al. 2018 chapter 3), *H. ruspolii* is more primitive than *H. gribodoi* and *H. araujoi.*

The colour of batumen used for sealing cracks varied between the *Hypotrigona* species. It was dark brown in *Hypotrigona ruspolii*, reddish-brown in *H. araujoi* and yellowish-white in *H. gribodoi*. We suggest that this could be as a result of differential preferences for resin sources between the species.

In conclusion, nesting sites, nest entrance architecture, brood cells arrangement and size of storage pots differ significantly between *H. gribodoi, H. araujoi* and *H. ruspolii*. Therefore, nest architecture can be used to positively identify these three *Hypotrigona* species in the field. Identification in the field will allow tailor made conservation efforts of *H. araujoi* and *H. ruspolii* since they nest in cavities in living trees in the forest which makes them vulnerable to deforestation. Farmers will use the tools to identify the bees in field and in their meliponiaries for domestication.

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CHAPTER 3

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

Abstract

Stingless bees are essential 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 *Hypotrigona* is extremely difficult due to many similarities among species. This has resulted 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 showed 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 clearly separates all 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 that there are cryptic species within this species complex, and that the *H. gribodoi* collected from Mwingi is potentially a new putative species. Thus, the use of morphometrics and molecular taxonomic approaches DNA barcoding in particular provide a convenient, robust and reliable way to identify *Hypotrigona* species. It also indicates the need for a thorough revision of *Hypotrigona.*

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Introduction

Stingless bees (Hymenoptera: Apidae: Apinae) are important pollinators of crops and wild plants (Heard 1999; Kiatoko et al. 2014; Slaa et al. 2006) and are therefore a major complement to honey bee pollination (Cortopassi-Laurino et al. 2006; Vanbergen 2013). Stingless bees produce honey that is different from that of honey bees due to its high moisture content (31%) compared to 20.2% in honey from honey bees. The high water content is enabled by the enzymes and other substances that are associated with antibiotic activity in the stingless bee honey (Lubertus et al. 2006). Although stingless bee's honey is produced in smaller quantities when compared to that of honey bees (*Apis mellifera*) (Kiatoko et al. 2016), their honey fetches higher prices due to its putative medicinal value (Kumar et al. 2012). Other stingless bee hive products include propolis and cerumen, both of which have been shown to have antioxidant activities (Pérez- Perez et al. 2013); antibacterial, immunomodulatory (Liberio et al. 2011; Temaru and Shimura 2007); anti-inflammatory effects (Araujo et al. 2012). The honey is said to inhibit dermal carcinogenesis in rodents (Pereira-Filho et al. 2014) and thus could be utilised in medicine. These commercial opportunities, combined with their ability to pollinate important plants have led to an increased interest in their commercial production. Their exploitation is however limited by lack of basic biological knowledge, and the ability to easily distinguish species. Moreover, taxonomic clarity is paramount for understanding pollinator ecology, especially in understudied areas like Africa (Archer et al. 2014).

There are two tribes of eusocial bees, the Apini (stinging bee) and Meliponini (stingless bees) (Michener 2007). Stingless bee species are differentiated from other bees by a reduced sting, reduced 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). Stingless bees of Africa are grouped into six genera (Eardley 2004); *Dactylurina* Cockerell (1934a), *Meliponula* Cockerell (1934a), *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 five genera collect their own food from wild flowers and commercial crops (Eardley 2004). African Stingless bees have been poorly studied, with the result that the classification of the group is still largely unresolved (Eardley 2004; Michener 2000). *Hypotrigona*, the focus of this study contains four species, *H. gribodoi, H. araujoi, H. ruspolii* and *H. squamuligera,* of which the latter only occurs in West Africa.

Hypotrigona are extremely difficult to identify due to similarities in their 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 (1961a) separated *H. gribodoi* and *H. araujoi* based on whole body length ratio. Eardley (2004) generated a taxonomic key in which he showed that the character differentiating the three *Hypotrigona* species. 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) showed that the head and scutal vestiture in *Hypotrigona* are weakly pinnate with puntuated scutal that are slightly shiny. Despite these attempts to differentiate *Hypotrigona* species, it remains difficult to identify these species without the expert taxonomic knowledge needed to interpret the keys. Therefore, robust tools that are easier to use are needed to differentiate between *Hypotrigona* species.

This study, therefore, combines morphometrics and DNA barcoding in an attempt to identify and differentiate the species of *Hypotrigona* found in Kenya. DNA barcoding tools have been used previously to identify bees; (Hurtado-Burillo et al. 2013; Sheffield and Hebert 2009; Magnacca and Brown 2012). Recently five Kenyan stingless bees were 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 cytochrome c oxidase 1 (*CO1*) was chosen as the focal region because it is bordered by two universal primers that work for a wide range of metazoans (Puillandre et al. 2012; Hebert et al. 2003) and has been shown to be most informative for species identification (Hajibabaei et al. 2007; Sheffield and Hebert 2009). The aim

of this study therefore was to apply a DNA barcoding protocol based on the *COI* gene sequence and morphometric analysis to identify the species of *Hypotrigona* found in Kenya.

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 (Fig. 3.1). Kakamega forest is a tropical rain forest in western Kenya (0°09'N to 0°22'N; 34°50'E to 34°58'E), supporting high biodiversity (Zimmerman 1972) including bees (Nkoba et al. 2012; Kasina et al. 2009). Whilst 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 et al. 2010).

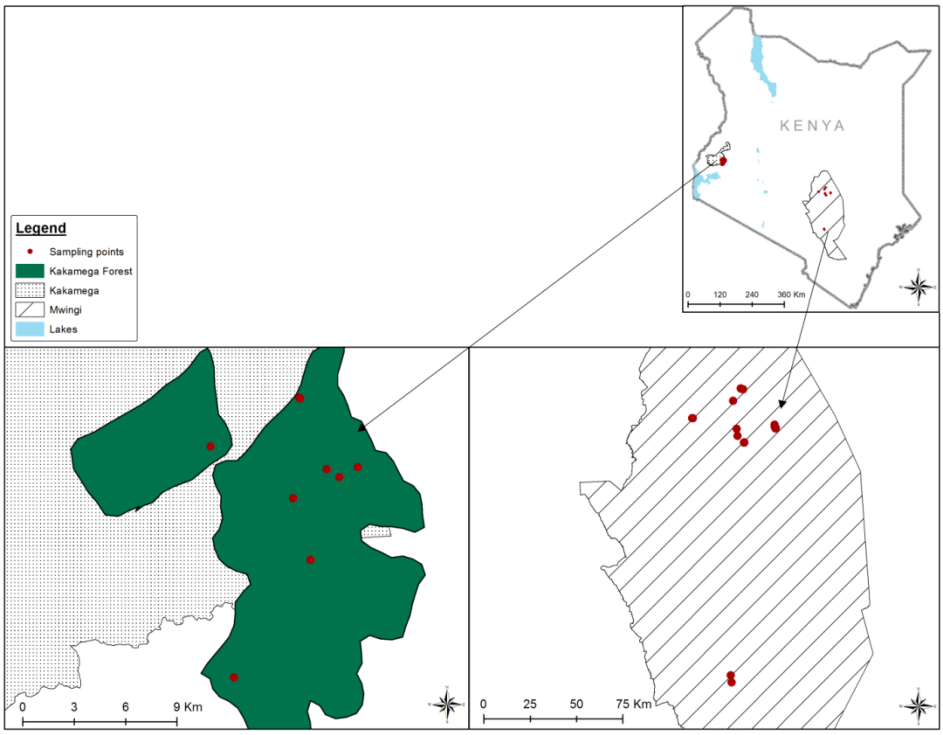

Fig. 3.1 Map of Kenya showing the two sampling areas, Kakamega forest (green) and Mwingi (chequered box). Circles represent sampling points where colonies were collected (ndungu et al. 2017).

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. squamuligera* occurs only in West Africa and could not be collected, this study included only three of the four 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's 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 and Engels 1992; Quezada-Euán et al. 2007). Each measurement was taken in triplicate (to an accuracy of 0.001 mm). Measurements included length of the forewing (WL) and its width (WW), distances between selected forewing veins, V3–V8, tibia length (TL), tibia width (TW), and femur length (FL) (Fig. 3.2a and 3.2b, See Appendix V). Voucher specimens are preserved at the museum of the African Reference Laboratory for Bee Health ICIPE in Nairobi, Kenya.

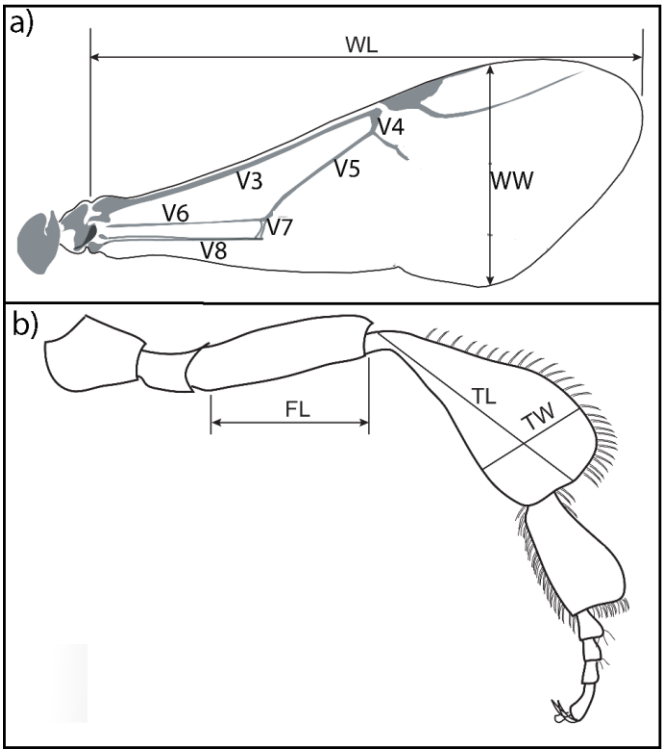

Fig. 3.2 Schematic representation of the right forewing and the right hind leg presenting the morphometric characters of interest. (a) Right forewing showing veins, WL= wing length; WW= wing width; V3= Marginal vein (R); V4= radial sector (RS); V5 = basal vein (M); V6= medial–cubital vein (M+Cu); V7= cubitus (Cu); V8= V. (b) Right hind leg. 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 procedures 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 and 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 et al. 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 visualised 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 analyser. The *COI* sequences were submitted to the Barcode of Life database (BOLD) and GenBank (Appendix I, Table 3.1).

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 for forewing and leg measurements were plotted. The log-transformed data were also subjected to Canonical Variate Analysis (CVA) to analyse 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

The 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 characterises 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 neighbour 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 neighbour for the species. In order 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 and Hebert 2007).

Phylogenetic Analyses

Bioedit (Hall 1999) was used to assemble and edit the sequences, and alignment done using Muscle, (Edgar 2004), and in MEGA 6 (Tamura et al. 2013) with default settings, and then converted into Phylip format using Seaview (Gouy et al. 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 neighbour-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 1993) 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 specimens of *Meliponula lendliana* from the BOFAS project in BOLD were used as outgroups (KU146611 and KU146608).

Results

Morphometrics

In the PCA plot, *H. ruspolii* separated completely from *H. araujoi*; however, *H. gribodoi* overlaped with *H. ruspolii* and *H. araujoi*. *Hypotrigona gribodoi* from Mwingi and *H. gribodoi* from Kakamega formed a single cluster. PC1 and PC2 contributed 54.4% and 13.4%, to the total variation in the separation of the species (Fig. 3.3a). Tibia width and length showed the highest contribution in PCA 1 to the differentiation of the species (0.5 and 0.417). Separation was greater in a CVA plot that sought to find maximal differences among *a priori* defined groups; CV1 and CV2 accounted for variances of 78.9% and 16.7% respectively, (Fig. 3.3b). Three clusters were formed; a) *H. araujoi*, 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 was 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). D^2 between *H. araujoi* and *H. gribodoi* from Kakamega was larger than that between *H. araujoi* and *H. gribodoi* from Mwingi (21.83 and 12.83).

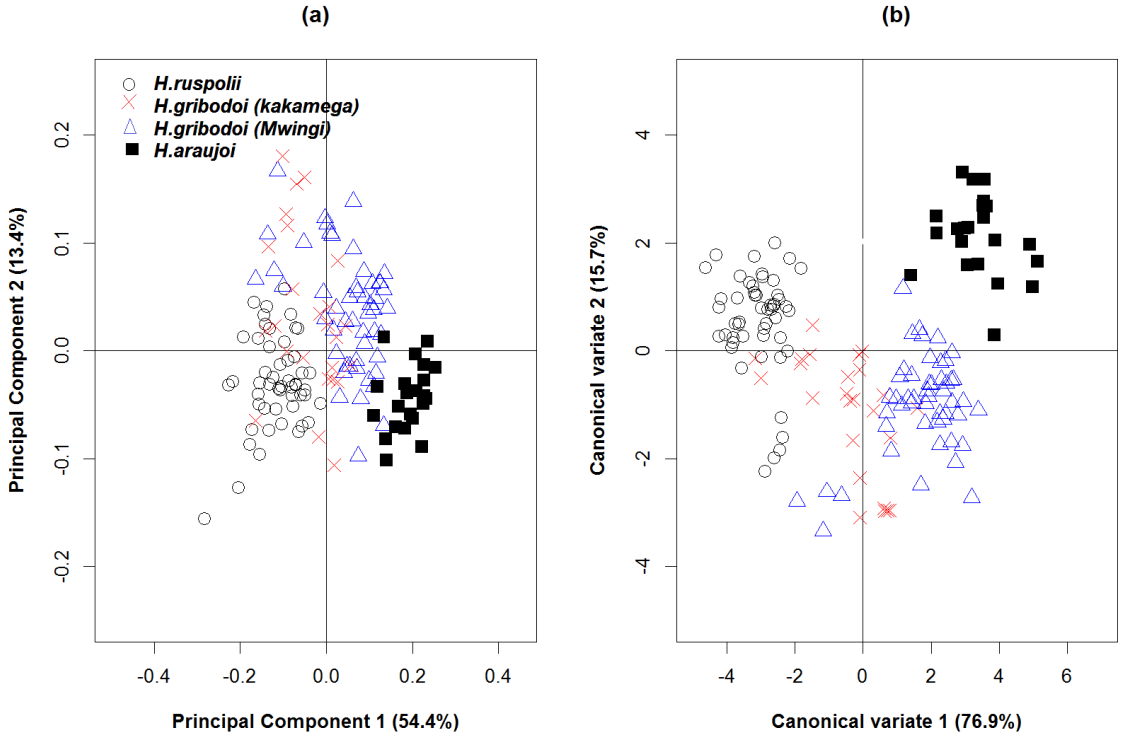

Fig. 3. 3 Multivariate analyses of the wing morphometric measurements. (a) PCA performed on *Hypotrigona*. PC1 and PC2 contributed 67.8% (54.4% and 13.4%), to the separation. *H. araujoi* partially separated from *H. gribodoi* and *H. ruspolii* (b) CVA performed on the complete dataset with the *Hypotrigona*. CV1 and CV2 contributed 93.6% to the separation (76.9% and 16.7%). *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. 3.4). In terms of the barcode gap analysis, the mean intraspecific distance within each species was 1.46 ± 0.19%, while the mean distance to the Nearest Neighbor (NN) was 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% (Table 1). BOLD calculated genetic mean distance within species and genus were 1.76% and 7.08%.

Table 3.1. DNA Barcode Gap Analysis of the three *Hypotrigona* species

Species	Mean Intraspecific (%)	Maximum Intra specific (%)	Nearest Species	Distance to Nearest Neighbour(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

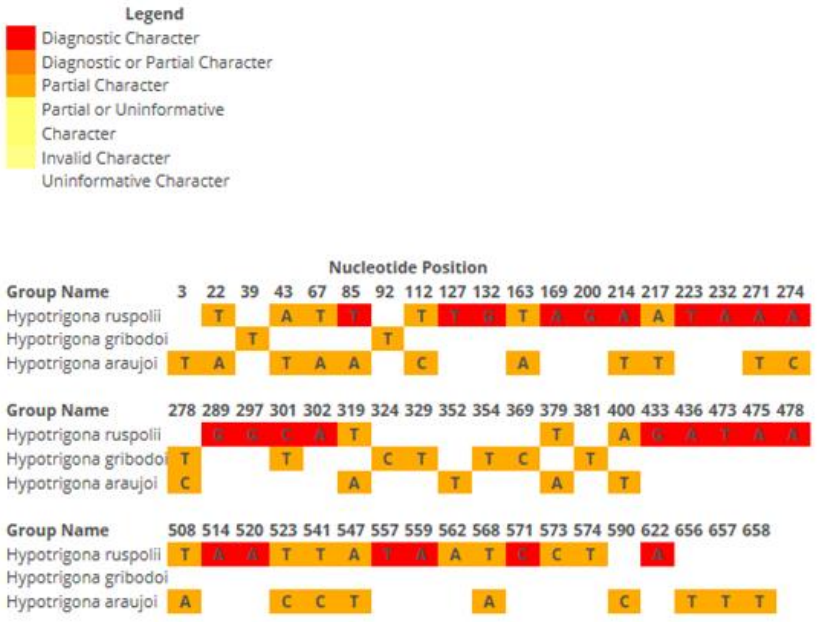

Fig. 3. 4. Characters that differentiate *H. gribodoi*, *H. ruspolii* and *H. araujoi*, generated with BOLD analysis tools. *Hypotrigona ruspolii* had 25specific diagnostic characters 25 and 17 partial diagnostic character while *H. araujoi* and *H. gribodoi* had 25 and 9 partial characters. The Individual sequence length = 658. No. groups in MSA with minimum 3 sequences = 3. Legend: *= diagnostic character; P= partial character.

Phylogenetic and distance analysis

The ML and Neighbour Joining methods supported the monophyly of *Hypotrigona* (99/100% bootstrap support (Fig. 3.5). *Hypotrigona ruspolii* is a well-supported monophyletic species (99/100% bootstrap support) and is sister to all the other species.

H. gribodoi from Kakamega and *H. araujoi* form paraphyletic clades (99/98% bootstrap support) and are sisters to *H. gribodoi* (Mwingi) (93/79% bootstrap support). *Hypotrigona gribodoi* from Mwingi forms a monophyletic, albeit poorly supported, clade (58/-% bootstrap support). 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 distance was in *H. araujoi* at 0.017 (1.7%), followed by *H. gribodoi* from Mwingi at 0.0084 (8.4%), and last *H. gribodoi* from Kakamega at 0.0021 (0.21%) (Appendix I, Fig. 1).

Discussion

Reduced wing venation was observed in all *Hypotrigona* species, a characteristic common in stingless bees (Wille 1983). In addition, the tibial width and length contributed to the highest differentiation between the species. These results are supported by those of Eardley (2004) where *H. ruspolii* was shown to have the narrowest tibia amongst the *Hypotrigona* (Eardley 2004). 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*. *Hypotrigona araujoi* was shown to have a wider tibia compared to *H. gribodoi* (Michener 1959).

DNA barcoding results were in contrast to those of 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 the 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 genus.

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 to *H. gribodoi* from Mwingi. *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, 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. 3.1). Such results have been observed in butterflies where closely related but morphologically and ecologically distinct species differed by only one to three nucleotides (Burns et al. 2007). The high intraspecific variation within *H. gribodoi* from Mwingi and those 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 the stingless bee *Plebeia remota* where the samples ecological characteristics differed significantly when collected from two different localities in Brazil. It was suggested that paleogographic and paleoclimatic events led to isolation of the two populations (Cristina et al. 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 et al. 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 morphometrics as tools for differentiating the three *Hypotrigona* species.

The data suggest a likelihood of cryptic species within *H. gribodoi* and thus a potentially new putative species from Mwingi. Mwingi and Kakamega are very different in terms of climatic conditions and are far apart, potential reasons for the high intraspecific genetic distances observed (Cristina et al. 2006). Kakamega is a tropical rainforest located in the highlands of western Kenya. It lies between 1500 - 1600 m above sea level (Tsingalia and 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 - 34^{oC} (Njoroge et al. 2010; Opiyo et al. 2011). Large areas of Mwingi are grasslands with shrubs, mainly dry land vegetation (Kaloi et al. 2005).

The *H. gribodoi* populations in Mwingi and Kakamega are isolated by a large distance, which includes the Great Rift Valley. Therefore, the posibility of interbreeding between these two *H. gribodoi* populations is unlikely. A study carried out on *Melipona subtinida* from Brazil 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, about fifty to few hundred meters between conspecific colonies (Roubik 2006). More specifically, *Hypotrigona* species mate about 100m around their nests (Portugal-Araujo and Kerr 1959) and they are known to forage across short distances (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 species and thus a potentially new putative species should be described. Use of additional molecular markers such as microsatellites in future studies will give a better understanding of *Hypotrigona* population genetics, population dynamics, biogeography, possible introgression, and evolution.

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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

Fig. 3.1. RAxML phylogram, bootstrap values for both 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

CHAPTER 4

Compounds extracted from heads of African stingless bees (*Hypotrigona*) as a prospective taxonomic tool

Abstract

Stingless bees are crucial pollinators of plants, and producers of honey. Species within the African stingless bee genus *Hypotrigona* are difficult to identify and differentiate due to morphological similarities. Chemical profiles of whole head extracts from workers of three *Hypotrigona* species: *H. gribodoi*, *H. araujoi* and *H. ruspolii* were studied by gas chromatography-mass spectrometry. A total of 50 components belonging to six chemical classes: hydrocarbons, aldehydes, alcohols, terpenoids, steroids and fatty acids were identified. Twenty-nine compounds were found in *H. araujoi*, 26 in *H. gribodoi* and 33 in *H. ruspolii* head extracts. Hydrocarbons, alcohols and fatty acids were the major classes, whilst steroids and terpenoids were minor. Aldehydes were found only in *H. ruspolii* while terpenoids were only present in extracts of *H. gribodoi* and *H. araujoi*. Eight chemical compounds were specific to *H. araujoi*, six to *H. gribodoi* and nine to *H. ruspolii*, showing both qualitative and quantitative differences. Workers were successfully grouped into their respective colonies and species using their chemical profiles. This study shows that, head extracts can be used as a reliable taxonomic tool for identifying and discriminating *Hypotrigona* species.

Key words: Speciation, head secretions, *Hypotrigona* species, chemical profile

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Introduction

Stingless bees (Apidae; Meliponini) are essential pollinators (Kakutani et al. 1993; Heard 1999) with over 500 identified species in 23 genera worldwide (Michener 2007). However, in Kenya, only 12 species of stingless bees belonging to six genera are known to date (Eardley 2004; Nkoba et al. 2012), with few studies targeting them over the years. Stingless bees produce honey with high medicinal value and thus fetch prices twice as much as honey from honey bees (Kiatoko et al. 2016). Stingless bees are reported to have complex communication systems that are regulated by cephalic secretions from both mandibular and labial glands (Engels et al. 1990). These secretions contain specific pheromones or kairomones (Gracioli-Vitti et al. 2012) that induce either defensive or aggressive behaviour just like in other eusocial insects (Blum and Brand 1972; Le Conte and Hefetz 2008; Yusuf et al. 2015). Secretions from labial glands of stingless bees are known to act as trail pheromones used in recruiting nest mates to food sources (Free 1987; Jarau et al. 2006; Schorkopf et al. 2007). On the other hand, secretions from the mandibular glands have been found to serve as alarm or repellent substances that play a role in interspecific and intraspecific defence (Schorkopf et al. 2009). Secretions from the mandibular glands of workers mainly contain hydrocarbons, alcohols, esters, acetones, ketones, carboxylic acids, and aldehydes (Engels et al. 1997; Schorkopf et al. 2009) which vary with life stages and between castes (Gracioli-Vitti et al. 2012). Cephalic secretions show similar chemical profiles both within species (Lopez et al. 2002) and between closely related species (Francke et al. 2000). Differences in chemical compositions of cephalic extracts between sex and castes (Francke et al. 2000) as well as with age (Engels et al. 1993; Poiani et al. 2014) are attributed to variations in volatile signals which enable workers to discriminate between nest and non-nest mates (Francke et al. 2000). Indeed, there were differences in cephalic secretions from 11 Brazilian social stingless bee species in the *Tetragonisca* and *Frieseomelitta* genera (Lopez et al. 2002)

Chemical compositions of the mandibular glands, as well as those of the cephalic extracts from some species of Neotropical stingless bees like *Scaptotrigona postica* and *Frieseomelitta* species have been studied extensively (Patricio et al. 2003; López

et al. 2002; Cruz-López et al. 2005). However, little is known of the cephalic secretions from African stingless bees (Leonhardt 2017).

Hypotrigona are small stingless bees (2mm in size) that are morphologically similar, thus making the species difficult to identify and differentiate (Eardley 2004). The *Hypotrigona* consists of four species; *H. gribodoi, H. araujoi, H. ruspolii* and *H. squamuligera* of which the latter is only found in West Africa (Eardley 2004). Several studies have attempted to distinguish *Hypotrigona* species using various approaches. This includes, Moure (1961) who separated *H. gribodoi* and *H. araujoi* based on whole body lengths. This was further confirmed through breeding experiments by Michener (1959) who demonstrated that *H. araujoi* and *H. gribodoi* could not mate and was indeed two separate biological species. In addition, Eardley (2004) used morphology of the legs, wings, head and thorax of workers to describe and differentiate the species. Despite the many attempts to differentiate *Hypotrigona* species, it still remains difficult to identify these species without the required expert taxonomic knowledge often needed to interpret the various taxonomic keys. Therefore, there is an urgent need to develop other tools which can be used to identify and correctly differentiate *Hypotrigona* species.

The aims of this study were to first identify and compare the chemical compositions of the extracts from whole heads of the workers of *Hypotrigona* in Kenya. Second, the study documents chemical components of head extracts that can be used to reliably differentiate these species.

Materials and methods

Bees: Workers of two of the three *Hypotrigona* species; *H. araujoi,* and *H. ruspolii* were collected from queen right colonies in Kakamega forest, Western Kenya whilst workers of *H. gribodoi* were collected from Mwingi, Eastern Kenya (Fig. 4.1). For each species, at least three workers were sampled per colony from three colonies, bringing the total number of samples analysed to 31.

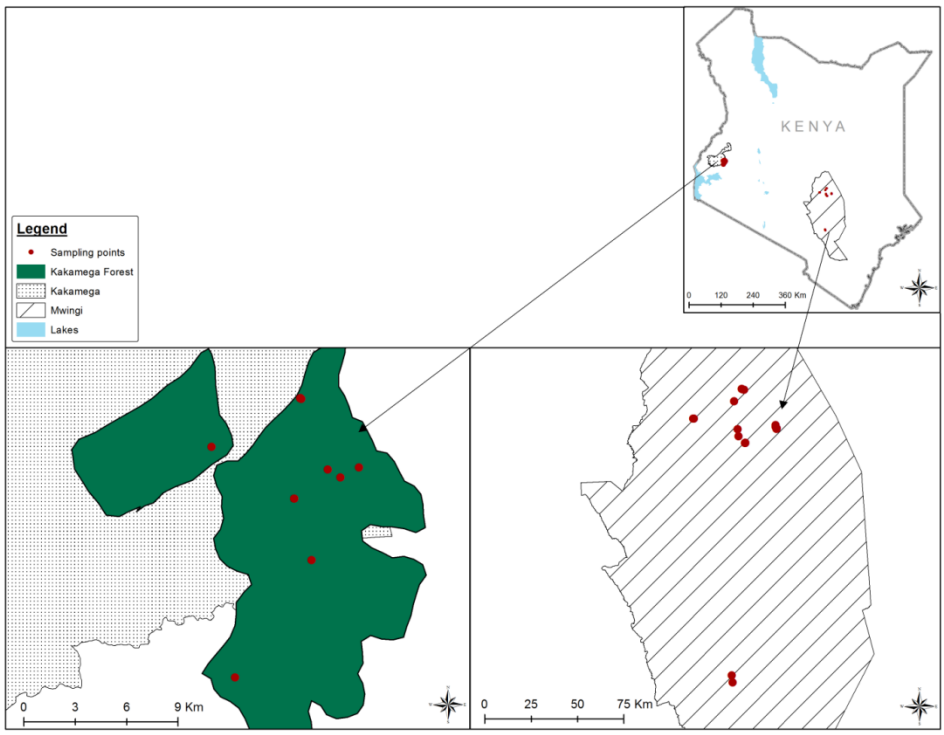

Fig. 4. 1 Map of Kenya showing the two sampling areas, Kakamega forest (green) and Mwingi (chequered box).

Head extracts: Bees were immobilised on ice, decapitated and their heads placed into clean pre-labelled 2ml sample vials containing 200µl HPLC grade dichloromethane. Samples were transported to the Department of Zoology and Entomology, University of Pretoria where they were stored at -20ºC until required for analysis. For analysis, 100µl of each head extract was placed into a 150µl Gas Chromatograph (GC) vial insert and concentrated under a gentle stream of clean nitrogen gas by removing the solvent. Ten (10) µl of GC grade N,O–Bis(trimethylsilyl) trifluoroacetamide **(**BSTFA) derivatising agent and 10µl of an internal standard mixture (containing ~1mg of *n*-Heptadecane and ~1mg Hexadecanoic acid) were added. To ensure complete derivatisation of the analytes, the mixture was allowed to stand for 4 hours in a refrigerator. This derivatisation process was facilitated by the addition of BSTFA which allows the formation of trimethylsilyl (TMS) derivatives of non-volatile compounds, like fatty acids and steroids thus, making them volatile enough to be analysed by a GC. While addition of an internal standard allowed for quantification of the individual chemical components relative to the mass ratios (RMR) and peak areas of the internal standards.

Gas Chromatography Mass spectrometer (GC-MS) analyses: One microlitre of each derivatised head extract was injected into a Schimadzu QP2010 Ultra GC-MS and analysed in the Electron Impact ionization (EI) mode on an Inert Cap 5MS/NP capillary column (30 m×0.25 mm×0.25 μm; GL Sciences, Tokyo, Japan). The oven was programmed as follows: 120°C for 5 min increased to a final temperature of 300°C at 20°C min^{-1} and held for 15 min bringing the total runtime per sample to 29 minutes. This allowed for all the components as well as the derivatives to come off the column. Helium was used as the carrier gas at a constant flow rate of 1.0 ml min^{-1}. The ion source was operated at 200°C with an interface temperature of 250°C, and mass spectral recorded between 40–600 m/z at 70 eV with a scan speed of 2500. Compounds were identified based on the comparison of mass spectra with those in published mass spectral libraries [NIST11 and Wiley (10th edition)], and an in-house spectral database of derivatised compounds. They were further confirmed using synthetic standards, diagnostic ions and Retention Indices as applicable (Appendix II Table S1).

Chemicals: Authentic synthetic standards for hydrocarbons, tridecanoic, tetradecanoic and pentadecanoic acids (purity ≥99%) were purchased from Altech Associates Inc. IL, US. Octatriacontanoic acid standard was purchased from ChemTik, Germany, while all the other fatty acids, steroids and terpenoids (purity ≥98%) were purchased from Sigma-Aldrich GmbH, Germany. Octadecanal was purchased from Albany International and the solvent dichloromethane (HPLC grade Chromsolv®) with a purity of ≥ 99.8% from Sigma-Aldrich.

Determination of double bond positions in unsaturated hydrocarbons: The position of double bonds in unsaturated hydrocarbons were determined using the dimethyl disulphide (DMDS) technique of Carlson et al. (1989) modified by Fombong et al. (2012). The procedure involved the addition of 100µl DMDS to an aliquot (100µl) of the head extracts followed by the addition of a 10µl 30mg/ml iodine solution (prepared in diethyl ether) to catalyse the reaction. The mixture was then heated for 12h at 50^{o}C. To neutralise the I_2, 10µl of 0.5M aqueous sodium thiosulphate was added to the mixture. The supernatant, containing DMDS adducts was decanted and analysed by GC-MS.

Qualitative and quantitative analysis of chemical profiles: Upon identifying the chemical compounds, the qualitative chemical profiles for each species were established using only those compounds that are present in > 90% of the samples for that species. These compounds were further grouped into the following chemical classes; hydrocarbons, aldehydes, alcohols, terpenoids, steroids, fatty acids and unknowns (those compounds that returned no library hits).

Quantitative chemical profiles were established using the peak area of each component relative to the peak area and relative mass ratio (RMR) of the appropriate internal standard. Concentrations of hydrocarbons, alcohols and terpenoids were computed relative to the RMR of *n*-heptadecane. On the other hand, those of fatty acids, aldehydes and steroids were computed using the RMR of hexadecanoic acid. All concentrations were expressed as microgram (µg) per bee head. Amounts for each chemical class were used to determine the relative proportions of these components as a percentage of the total concentration.

Statistical Analyses

Unless otherwise stated, results are presented as means ± standard errors of means (SEM) of individual chemical components in the total extract per bee head. Differences between chemical classes were tested using Analysis of Variance (ANOVA) and post hoc analysis with Tukey HSD test. A non-Metric Multidimensional Scaling (n-MDS) with Bray-Curtis dissimilarity was used to visualise chemical disparity among the samples coding for both species and colonies. All analyses were carried out using SAS 9.4 (SAS Institute Inc., USA).

Results

Chemical profiles from head extracts of *Hypotrigona* species

Fifty (50) compounds were identified, 48 of which belong to six (6) chemical classes. These consist of hydrocarbons of chain lengths C18 to C34 mainly alkanes and alkenes; monohydric, poly and unsaturated aliphatic alcohols, an aldehyde; saturated and unsaturated (ω-3 to ω-9) fatty acids; mono and sesquiterpenes, steroids and two unidentified compounds (Table 4.1 and Fig. 4.2).

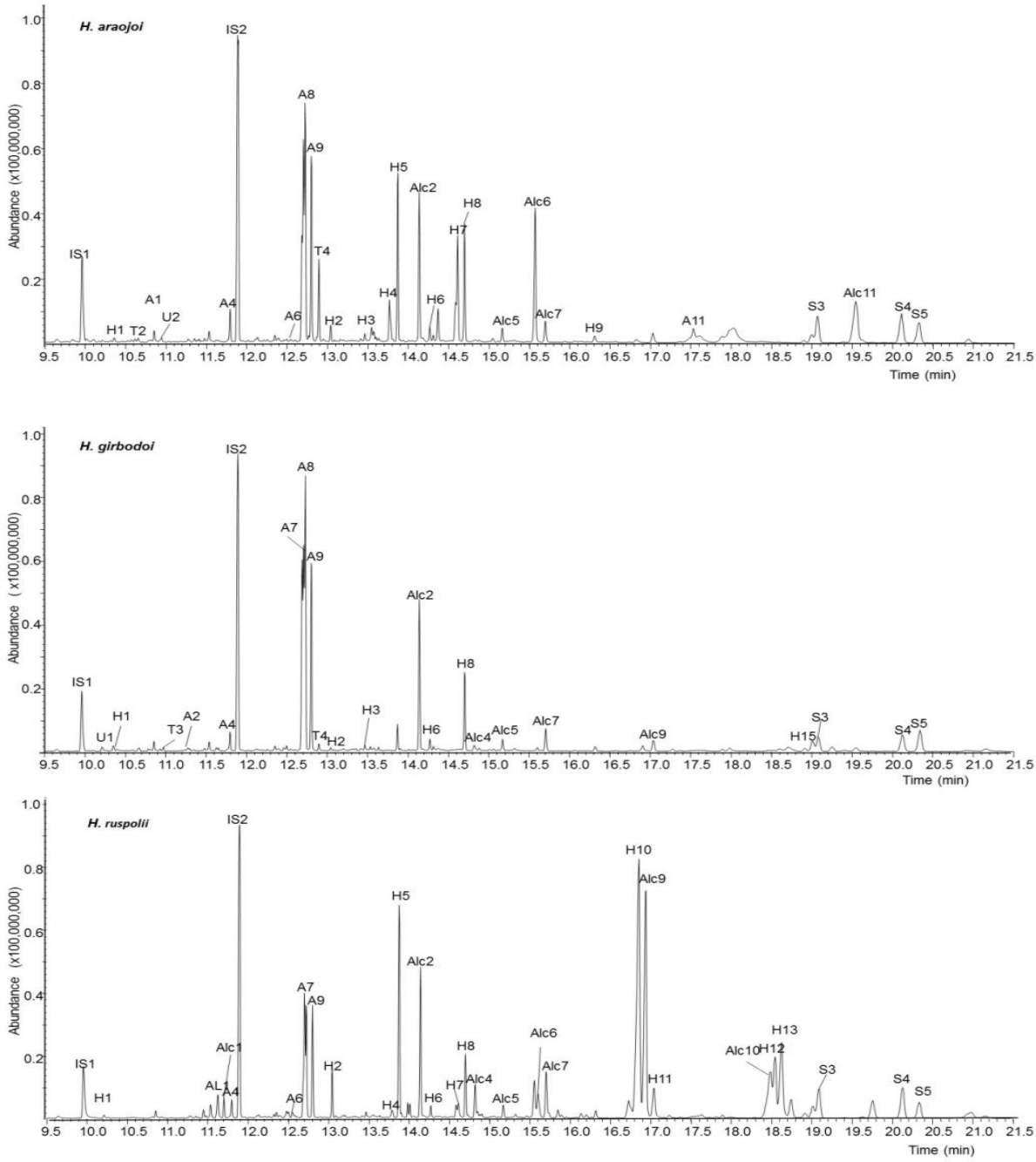

Fig. 4.2 Representative total ion chromatograms (TICs) of the chemical profiles from head extracts of *Hypotrigona araujoi*, *H. gribodoi* and *H. ruspolii* workers. IS1 and IS2 are the internal standards, namely Heptadecane and Hexadecanoic acids. Classes and individual compounds are identified by their IDs as listed in Table 1 where A = Fatty acids, AL = Aldehyde, Alc = Alchohols, H = Hydrocarbons, S = Steriods, T = Terpeniods and U = Unidentified compounds.

Qualitative chemical profiles from the head extracts of *Hypotrigona* species

Twenty-nine compounds were identified from the head extracts of *H. araujoi*, 26 from *H. gribodoi* and 33 from *H. ruspolii*. Profiles of *H. araujoi* and *H. gribodoi* contained five representative classes of compounds (hydrocarbons, alcohols, fatty acids, steroids, terpenoids as well as unidentified compounds) (Table 4.1). While those of *H. ruspolii* contained only four classes, including an aldehyde with no terpenoids and unidentified compounds (Table 4. 1).

In terms of percentage proportion for the classes of compounds, profiles of *H. ruspolii* mainly contained hydrocarbons (43.09 ± 7.46%) and alcohols (29.12 ± 7.76%), with small proportions of steroids and no terpenoids. *Hypotrigona araujoi* and *H. gribodoi* head extracts predominantly contained fatty acids (42.82 ± 7.27% and 45.47 ± 8.35%); and hydrocarbons (24.50 ± 3.00% and 27.22 ± 4.20%) and minor proportions of terpenoids. The major chemical compounds found in these three *Hypotrigona* species were *n*-tricosane, *n*-heptacosane and octadecanoic acid (Table 1). In addition, pentacosanol, tricosanol and 1-nonacosanol occurred in major proportions in *H. araujoi, H. gribodoi* and *H. ruspolii.* Moreover, octadeca-9,12,15-trienoic acid and *Z, Z* octadeca, 9,12-dienoic acid were present in high proportions in extracts from *H. araujoi* and *H. gribodoi* (Table 4.1).

Table 4. 1 Proportions (% ± SEM) of individual and classes of compounds identified from head extracts of the three *Hypotrigona* species

ID	RI	Hydrocarbons	*H. araujoi* (n = 10)	*H. gribodoi* (n = 12)	*H. ruspolii* (n = 9)
H1	1800	*n*-Octadecane*	0.62 ± 0.20	-	-
H2	2000	*n*-Eicosane	1.01 ± 0.11	0.47 ± 0.10	2.61 ± 0.30
H3	2200	*n*-Docosane	0.80 ± 0.10	1.85 ± 0.94	-
H4	2332	*Z* (9)-Tricosene	2.61 ± 0.57	-	0.85 ± 0.07
H5	2342	*n*-Tricosane	7.10 ± 0.68	5.37 ± 0.56	8.32 ± 1.05
H6	2497	*n*-Tetracosane	1.53 ± 0.20	1.42 ± 0.23	0.62 ± 0.07
H7	2738	*Z* (9)-Heptacosene	-	-	0.92 ± 0.10
H8	2764	*n*-Heptacosane	7.86 ± 0.73	11.51 ± 1.53	2.15 ± 0.17
H9	2845	*n*-Octacosane	1.20 ± 0.20	1.18± 0.20	0.30 ± 0.08
H10	2900	*n*-Nonacosane	1.77 ± 0.21	-	15.71 ± 2.79
H11	2912	1,37-Triacontadiene	-	4.27± 0.42	1.83 ± 0.22
H12	2996	Triacontene*	-	-	3.33 ± 0.55
H13	3044	*n*-Triacontane*	-	-	3.91 ± 0.63
H14	3200	*n*-Dotriacontane*	-	-	2.54 ± 1.41
H15	3300	*n*-Tetratriacontane*	-	1.15± 0.22	-
			24.50 ± 3.00	***27.22 ± 4.20***	***43.09 ± 7.46***
		Aldehydes			
AL1	1856	Octadecanal*	-	-	2.63 ± 0.62
					2.63 ± 0.62
		#Alcohols			
Alc1	1794	Hexadecanol*	-	-	2.06 ± 0.99
Alc2	1961	Octadecanol	0.60 ± 0.07	-	0.74 ± 0.17
Alc3	2253	1-Eicosanol*	-	-	0.62 ± 0.13
Alc4	2507	Tricosanol	-	5.15 ± 1.03	4.59 ± 3.22
Alc5	2575	Tetracosanol	1.70 ± 0.20	3.78 ± 0.27	0.69 ± 0.07
Alc6	2694	Pentacosanol	7.74 ± 1.27	-	1.39 ± 0.09
Alc7	2762	1-Heptacosanol	2.70 ± 0.26	-	2.50 ± 0.17
Alc8	2800	1-Octacosanol*	-	-	1.26 ± 0.26
Alc9	3074	1-Nonacosanol	-	1.98 ± 0.32	11.53 ± 2.10
Alc10	2798	1,30-Triacontanediol*	-	-	3.73 ± 0.56
Alc11	2998	1-Dotricontanol*	6.67 ± 1.20	-	-
			19.11 ± 3.00	***10.91 ± 1.62***	***29.12 ± 7.76***
		#Fatty acids			
A1	1822	Tridecanoic acid	0.81 ± 0.13	-	0.71 ± 0.28
A2	1841	Tetradecanoic acid*	-	0.36 ± 0.09	-
A3	1943	Pentadecanoic acid	-	0.39 ± 0.09	0.31 ± 0.06
A4	2041	Hexadecenoic acid	1.38 ± 0.14	1.33 ± 0.11	0.96 ± 0.19
A5	2134	Heptadecanoic acid*	0.37 ± 0.06	-	-
A6	2234	Octadeca-9,12,15-trienoic acid	18.93 ± 2.62	-	0.51 ± 0.13
A7	2209	*Z,Z* Octadeca, 9,12-dienoic acid	-	18.41 ± 3.32	8.59 ± 3.90
A8	2215	*E*-Octadec-9-enoic acid	-	12.98 ± 3.70	4.35 ± 1.38
A9	2207	Octadecanoic acid	10.52 ± 1.35	11.32 ± 0.87	5.75 ± 1.90
A10	2542	Heneicosanoic acid	1.38 ± 0.30	0.68 ± 0.17	-
A11	2732	Tricosanoic acid*	0.61 ± 0.19	-	-
A12	3808	Octatriacontanoic acid*	8.83 ± 2.48	-	
			42.82 ± 7.27	***45.47 ± 8.35***	***21.19 ± 7.83***
		#Steriods			
S1	3207	Desmosterol*	1.58 ± 0.19	-	-
S2	3255	Cholest-5-ene*	-	1.68 ± 0.31	-
S3	3263	Campesterol	3.41 ± 0.33	2.49 ± 0.28	0.62 ± 0.18
S4	3354	Beta Sitosterol	3.65 ± 0.47	3.76 ± 0.57	1.70 ± 0.60
S5	3286	Stigmasterol	2.69 ± 0.26	3.39 ± 0.42	1.66 ± 0.67
			11.33 ± 1.25	***11.31 ± 1.59***	***3.98 ± 1.45***

ID	RI	Hydrocarbons	*H. araujoi* (n = 10)	*H. gribodoi* (n = 12)	*H. ruspolii* (n = 9)
		#Terpenoids			
T1	1312	Citronellol*	-	0.58 ± 0.17	-
T2	1813	Farnesol*	0.23 ± 0.07	-	-
T3	1156	Isoborneol*	-	0.37 ± 0.10	-
T4	2529	Geranyllinalool	1.23 ± 0.29	3.37 ± 2.80	-
			1.47 ± 0.35	***4.32 ± 3.07***	**-**
		Unidentified			
U1	1520	Unidentified 1*	-	0.77 ± 0.20	-
U2	1762	Unidentified 2*	0.17 ± 0.07	-	-
			0.17 ± 0.07	***0.77 ± 0.20***	

ID = Peak identity on the chromatograph (Fig. 4.1) based on retention times, RI = Retention index of the compound, # = TMS derivatives, *= compound present in one species. Bolded and italicised values are mean proportions for each class of compound, - = absence/not detected. H = Hydrocarbons, Al = Aldehyde, Alc = alcohols, A = fatty acids, S= steroids, T = Terpenoids, U = Unknown compound.

The most abundant compounds in the profiles of *H. araujoi* were octadecanoic acid, *n*-tricosane and octadecanol (Fig. 4. 2). In *H. gribodoi*; E-octadec-9-enoic acid, Z, Z Octadeca, 9,12-dienoic acid, octadecanoic acid and octadecanol were the most abundant. While the profile of *H. ruspolii* was dominated by *n*-nonacosane, *n*-tricosane and 1-nonacosanol (Fig. 4.2).

Eight chemical compounds including *n*-octadecane, 1-dotricontanol, heptadecanoic acid, tricosanoic acid, octatriacontanoic acid, desmosterol, farnesol and unidentified compound 2 were specific to *H. araujoi* (Table 4. 1). *n*- Tetratriacontane, tetradecanoic acid, cholest-5-ene, citronellol, isobornel and unidentified compound 1 were specific to *H. gribodoi*. While Z-(9)-heptacosene, triacontene, *n*-triacontane, *n*-dotriacontane, octadecanal, hexadecanol, 1-eicosanol, 1-octacosanol and 1, 30-triacontanediol (Table 4.1) were unique to the profile of *H. ruspolii*.

Quantitative chemical profiles from head extracts of *Hypotrigona* species

The concentrations (µg) of fatty acids, alcohols, hydrocarbons and steroids varied between the *Hypotrigona* species. We found significant differences in the concentrations of hydrocarbons, aldehydes, alcohols and steroids from the head extracts of all the three *Hypotrigona* species (ANOVA, Wilks' $\lambda = 0.05$, $F(16,22) = 4.7744$, df =18, $P = 0.00045$) (Fig. 4.3). Further, Tukey HSD test shows significant difference in the concentrations of hydrocarbons ($P = 0.00591$); aldehydes ($P = 0.022$); alcohols ($P = 0.0263$) and steroids ($P = 0.0375$) between *H. araujoi* and *H. ruspolii*. Profiles of *H. araujoi* and *H. gribodoi* had higher amounts of fatty acids (3.76 ± 0.62 µg, 2.17 ± 0.30 µg) and hydrocarbons (2.01 ± 0.23 µg, 1.36 ± 0.24 µg)

with low amounts of terpenes. On the other hand, *H. ruspolii* had significantly high concentration of alcohols (5.92 ± 1.53 µg) and hydrocarbons (8.17 ± 1.85 µg), whilst steroids and aldehydes were recorded in low amounts (Fig. 4.3). Using n-MDS with Bray- Curtis dissimilarity, workers of the *Hypotrigona* species were successfully grouped into their species and colonies of origin (Fig. 4.4).

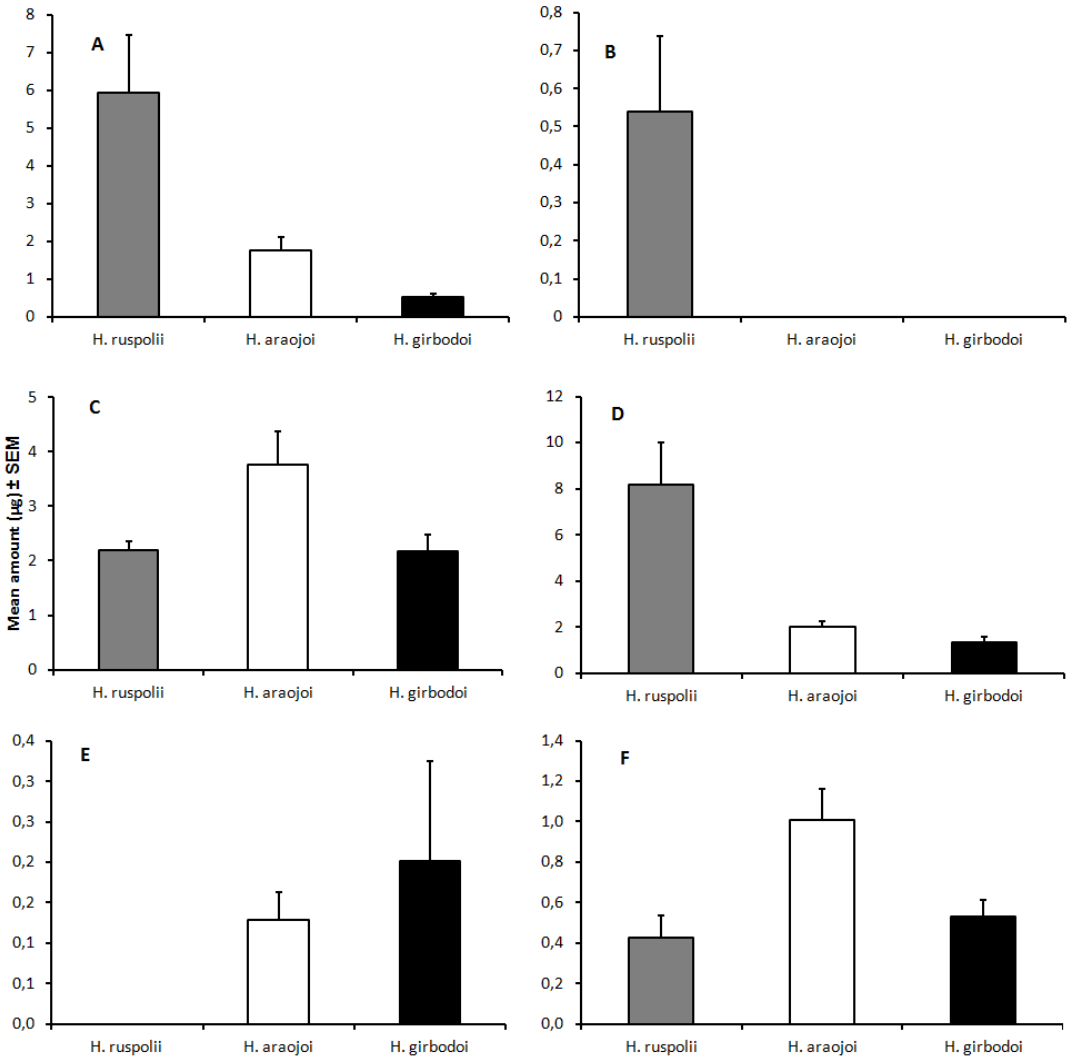

Fig. 4. 3 Concentration (mean ± SEM) of the classes of compounds identified from head extracts of *Hypotrigona ruspolii* (grey bars), *H. araujoi* (open bars) and *H. gribodoi* (black bars). A = Alcohol, B = aldehydes, C = Fatty acids, D = Hydrocarbons, E = Terpenoids and F = Steroids. Note the difference in the scale for the Y axes.

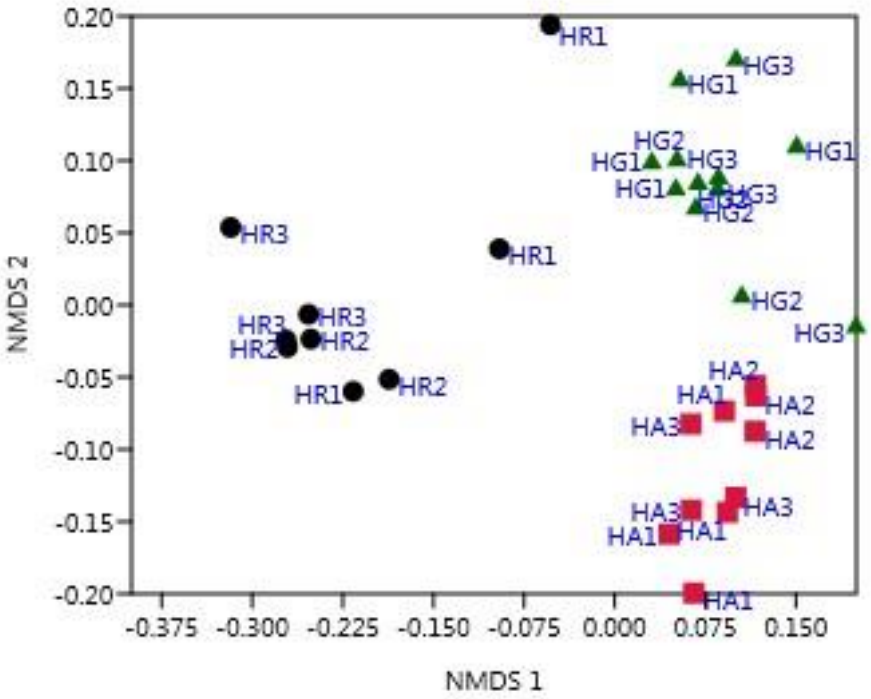

Fig. 4. 4 n-MDS plot of components 1 and 2 showing the separation of *H gribodoi*, *H. araujoi*, *H. ruspolii* into their respective species and colonies of origin. The species are represented by the first two letters followed by colony numbers. HR=*H. ruspolii*, HA=*H. araujoi* and HG=*H. gribodoi.*

Discussion

Differences were found in the chemical profiles of head extracts between three *Hypotrigona* species. A total of fifty (50) compounds, 48 from six chemical classes and two unknowns were identified. The profiles of *H. ruspolii* contained 33 compounds, whilst those of *H. araujoi* and *H. gribodoi*, contained 29 and 26 coumpnds repectively. Extracts from whole heads contain chemical compounds of both surface (cuticular) and glandular origin, including those of mandibular and labial gland secretions often used in social insects communication (Free 1987; Leonhardt 2017). The use of whole head extracts in chemical profile studies are often easier to compare than glandular dissections, since they are easy to access, requires little expertise and can be carried out by amateurs (Meulemeester et al. 2011). Furthermore, volatiles analysed from whole heads and dissected glands (mandibular or labial glands) of stingless bee in the genus *Frieseomellita* were found to have roughly the same quantities of cephalic secretions (López et al. 2002).

In this study, six classes of compounds, hydrocarbons, alcohols, fatty acids, terpenes, steroids and aldehydes were identified. Alkanes and alkenes were the two major hydrocarbons found in this study. Alkanes are used for water proofing and their levels could be affected by stages of development, i.e., nurse, foragers and guard bees; or changes in temperature and humidity. Alkenes are involved in communication in honey bees (*Apis mellifera)* (Dani et al. 2005). In addition, cuticular hydrocarbons have been reported to be used for communication in stingless bees (Leonhardt 2017) and a high diversity of alkenes have been reported in neotropical stingless bees (Martin et al. 2017) suggesting divergence in this chemical signature during speciation.

These three *Hypotrigona* species vary in their alkene profiles, suggesting that these alkenes could be used to distinguish between the species. Indeed, alkenes and fatty acids have been indicated to be utilised in nestmates recognition (Kather et al. 2011) in stingless bees and also in the termite raiding ant *Megaponera analis* (Yusuf et al. 2010).

Our results show high levels of alcohols in all species. Some alcohols such as 1-tetracosanol and tricosanol were common to all species, whilst others were specific. For instance, 1-dotricontanol was specific to *H. araujoi* while hexadecanol, 1-

eicosanol, 1-octacosanol and 1,30-triacontanediol were specific to *H. ruspolii.* The alcohols found in this study differ from those found in Neotropical stingless bees. In Brazillian stingless bees, 2-heptanol, 2-octanol, Z-5-tetradecenylbutanoate and Z-7-hexadecenylbutanoate were found in *S. postica* (Engels et al. 1997) whilst, 2-nonanol and 2-undecanol were detected in *Frieseomelitta* species (López et al. 2002). In contrast to Neotropical stingless bees in which 2-heptanol was found in five of the nine studied stingless bees (Leonhardt 2017) , this alcohol was absent in *Hypotrigona*, suggesting that it could have been acquired after the splitting of the African –Australian and neotropical clade of stingless bees (Rasmussen and Cameron 2010).

We found minor quantities of terpenoids in head extracts of *H. gribodoi* and *H. araujoi* while none was detected in *H. ruspolii*. Terpenes are oxygen-containing compounds and have been found in the secretions of cephalic glands of some Neotropical stingless bees (Francke et al. 2000; Cruz-López et al. 2001; Patricio et al. 2003; Cruz-López et al. 2005). Terpenoids are derived from plant resin, which are known to contain terpenes and are used for nest construction and defense (Leonhardt and Schmitt 2009). Here, geranyl linalool was common to two species *H. gribodoi* and *H. araujoi* whilst isoborneol and farnesol were specific and present only in *H. gribodoi* and *H. araujoi.*

The complete separation of the *Hypotrigona* species shows that chemical components can be effectively used in the taxonomic separation of the three species (see Fig 4.4) which are currently difficult to tease apart using morphological features.

In conclusion, the composition of the head extracts from the workers of three *Hypotrigona* species, i.e., *H. gribodoi, H. ruspolii, H. araujoi* are different in both their quantities and qualities. The chemical profiles are specific and therefore could be utilised in the identification of African *Hypotrigona* species.

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APPENDIX II

Table S1 Compounds from head extracts of *Hypotrigona* species and the methods used to identify each on the GC-MS.

ID	Diagnostic ion	Compound	Method of identification
H1	254	*n*-Octadecane	MS, RI, ST
H2	282	*n*-Eicosane	MS, RI, ST
H3	310	*n*-Docosane	MS, RI, ST
H4	322;DMDS:55, 97, 111	*Z* (9)-Tricosene	MS, RI, ST
H5	324	*n*-Tricosane	MS, RI, ST
H6	338	*n*-Tetracosane	MS, RI, ST
H7	378;DMDS:173,299,472	Z-(9)-Heptacosene	MS, RI, ST
H8	380	*n*-Heptacosane	MS, RI, ST
H9	394	*n*-Octacosane	MS, RI, ST
H10	408	*n*-Nonacosane	MS, RI, ST
H11	418	1,37-Triacontadiene	MS, RI
H12	420	Triacontene	MS, RI
H13	422	*n*-Triacontane	MS, RI, ST
H14	450	*n*-Dotriacontane	MS, RI, ST
H15	478	*n*-Tetratriacontane	MS, RI
AL1	43, 57, 69, 109, 268	Octadecanal	MS, RI, ST
Alc1	43, 55, 75, 103, 283,299	Hexadecanol	MS, RI, ST
Alc2	43, 75, 83, 97, 103,111, 227, 269,327	Octadecanol	MS, RI, ST
Alc3	43, 57, 75, 103, 111, 327, 339,355	1-Eicosanol	MS, RI, ST
Alc4	43, 57, 75, 103, 339,381, 397	Tricosanol	MS, RI
Alc5	43, 57, 75, 103,111, 395, 411	Tetracosanol	MS, RI
Alc6	43, 57,75, 103,111, 409, 425	Pentacosanol	MS, RI
Alc7	43, 57,75, 103,111, 395, 425, 437,453	1-Heptacosanol	MS, RI
Alc8	43, 57,75, 103,111, 395, 425, 482	1-Octacosanol	MS, RI
Alc9	43, 57,75, 103,111, 395, 425, 496	1-Nonacosanol	MS, RI
Alc10	43, 69, 82, 96, 111, 124, 138, 152, 292, 390, 418, 454	1,30-Triacontanediol	MS, RI
Alc11	43, 69, 82, 96, 111, 125, 138, 152, 294, 392, 466	1-Dotricontanol	MS, RI
A1	43, 55, 73, 117, 129, 145, 271, 286	Tridecanoic acid	MS, RI, ST
A2	43, 55, 73, 117, 132, 145, 285, 300	Tetradecanoic acid	MS, RI, ST
A3	43, 55, 73, 117, 129, 145, 285, 299, 314	Pentadecanoic acid	MS, RI, ST
A4	43, 55, 73, 117, 132, 145, 285, 269, 285, 299, 313, 328	Hexadecenoic acid	MS, RI, ST
A5	43, 55, 73, 117, 132, 145, 201, 257, 283, 299, 327, 342	Heptadecanoic acid	MS, RI, ST
A6	44, 95, 121, 149, 163, 177, 191, 205, 263,350	Octadeca-9,12,15-trienoic acid	MS, RI, ST
A7	41, 55, 67, 73, 81, 95, 109, 117, 129, 262, 337, 352	*Z,Z* Octadeca, 9,12-dienoic acid	MS, RI, ST
A8	41, 55, 67, 73, 81, 96, 110, 117, 129, 145, 264, 311,339, 354	*E*-Octadec-9-enoic acid	MS, RI, ST
A9	43, 55, 69, 73, 117, 132, 145, 201, 313,327, 341, 356	Octadecanoic acid	MS, RI, ST
A10	43, 55, 73, 117, 132, 145, 201, 339, 355, 383, 398	Heneicosanoic acid	MS, RI, ST
A11	43, 55, 73, 117, 132, 145, 201, 339, 355, 383, 426	Tricosanoic acid	MS, RI, ST
A12	69,83, 127, 180, 222, 265, 565	Octatriacontanoic acid	MS, RI
S1	41, 55, 69, 73, 75, 81, 95, 107, 119, 129, 145, 159, 253, 327,343, 351, 366, 372, 441, 456	Desmosterol	MS, RI, ST
S2	43,73,75,129, 441, 456, 531, 547	Cholest-5-ene	MS, RI, ST
S3	41, 43, 55/57, 73, 129, 343, 367, 382, 457, 472	Campesterol	MS, RI, ST
S4	41, 43, 55, 73, 129, 357, 381, 396, 471, 486	Beta Sitosterol	MS, RI, ST
S5	41, 43, 55, 69, 73, 83, 129, 255, 351, 355, 379, 394, 469, 484	Stigmasterol	MS, RI, ST
T1	73, 81, 95, 123, 143, 213, 228	Citronellol	MS, RI, ST
T2	41, 69, 73, 75, 93, 135, 143, 156, 189, 279, 294	Farnesol	MS, RI, ST
T3	75, 81, 95, 117, 147, 167, 191, 211, 269, 284	Isoborneol	MS, RI, ST
T4	41, 69, 81, 107, 136, 161, 189, 221, 247, 257, 290	Geranyllinalool	MS, RI, ST
U1	73, 75, 103, 129, 199, 217, 287,	Unidentified 1	
U2	73, 75, 99, 103, 129, 173, 259	Unidentified 2	

ID = the identity of peaks as represented in Fig. 4.1, MS = published mass spectra from MS libraries (NIST and Wiley), RI = retention index and ST = identification confirmed using synthetic standard compound.

CHAPTER 5

General Conclusion

Identification of *Hypotrigona* is difficult and ambiguous (Eardley 2004) and the use of available taxonomic key requires expertise. In this study, integrated taxonomic approaches using simple and robust tools were employed to identify three *Hypotrigona* species found in Kenya. The tools included; nesting sites and nest architecture, morphometrics, DNA barcoding and chemical profiling of secretions from the head of worker bees. Nesting site and nest architecture tools can be used by farmers to identify *Hypotrigona* species in their meliponiaries. Further, the tools can be used by field researchers to identify the species while collecting samples in meliponiaries and in the wild (see the key, Appendix III, page 89). In this Chapter, a summary of key findings from the three data chapters is given followed by recommendations and suggestions for future studies.

Nest sites and Nest architecture of the *Hypotrigona* species

In Chapter 2, the three *Hypotrigona* species differ in terms of nest sites and colour of their nest entrances. These distinguishing features can be applied by bee farmers and researchers for locating nests in the field. Such features like nest specificity had been reported by Roubik (2006) and Rasmussen and Camargo (2008). *Hypotrigona araujoi* and *H. ruspolii* nest in indigenous live trees in the forest while *H. gribodoi* nests in varying substrates including crevices in mud walls and rocks. The external nest entrances from wild and domesticated colonies were white or cream for *H. gribodoi,* yellowish-brown in *H. araujoi* and dark brown in *H. ruspolii*. These specific differences in colour are independent of the environmental conditions as observed following the transfer of the nests from Kakamega and Mwingi to ICIPE, Nairobi. Besides identification, nest entrances are important because they allow foragers to access the nest and at the same time allowing the guard bees to exclude intruders from robbing the resources (Grüter et al. 2010). A distinctive character observed in *H. gribodoi* colonies was the presence of an internal entrance tube. The tube extended from the external nest entrance into the hive and all the way to the storage pots. These results corroborate previous findings where internal entrances were observed in *H. gribodoi* nests in Ghana (Bassindale 1955). In this study, a

simple nest architecture key which can be used by farmers and field researchers was generated (Appendix III, page 89).

Brood cell arrangement was notably variable between the *Hypotrigona* species; the brood cells were clustered in *H. gribodoi* and *H. ruspolii*, while in *H. araujoi* they appeared in the form of comb-like structures in vertical layers. This tool can be easily applied for preliminary identification by farmers and field researchers. Clustering brood cell arrangement is a common feature among primitive bees (Kerr and Maule 1964; Wille 1964). Therefore, the comb-like nest structure in *H. araujoi* suggests that they are more advanced than *H. gribodoi* and *H. ruspolii*. This was further confirmed through mitochondrial *COI* gene sequences, which showed that *H. ruspolii* is more ancient than *H. araujoi* (Fig 3.1, Chapter 3, page 62).

In this study, specific nest architecture characteristics were identified for *H. araujoi* and *H. ruspolii*. *Hypotrigona araujoi* had distinctive strong pillars emerging from the brood (Figure 2.3, Chapter 2, page 28) whilst *H. ruspolii* had a characteristic dark brown outer involucrum covering the brood cells, honey and pollen pots. Construction of involucrum is an adaptation to maintain optimum temperatures for the growth of developing larvae in the brood cells (Barbosa et al. 2013) as well as providing protection to the brood cells from natural enemies such as ants (Figueiredo-Mecca et al. 2013; Rasmussen and Camargo 2008). Similar to clustering brood cells, involucrum has also been indicated in primitive stingless bees (Rasmussen and Camargo 2008). Hence, the presence of involucrum in *H. ruspolii* nests' further confirms that they are more ancient than the other *Hypotrigona* species.

Morphometrics and DNA barcoding

In Chapter 3, it was found that morphometrics and mitochondrial DNA (DNA barcodes) using *COI* gene can be applied as reproducible and convenient tools in large-scale sequencing to identify *Hypotrigona*. Morphometric analyses showed an overlap between *H. gribodoi* collected from Kakamega with *H. ruspolii* and *H. gribodoi* from Mwingi. Nonetheless, DNA barcoding can be used to identify *Hypotrigona* samples whose nest sites or nest architectural information are lacking. For a reliable identification of *Hypotrigona* species there is a need for combining

DNA barcoding and morphometrics as tools for differentiating the three *Hypotrigona* species. Integration of morphometric and DNA barcoding tools have been used to detect cryptic speciation in the stingless bee *Melipona yucatanica* (May-Itzá et al. 2010), to resolve the taxonomy of the western Malagasy stingless bee *Liotrigona moure* (Koch 2010) and for differentiation of the Neotropical bee *Melipona bechii* (Quezada-Euán et al. 2007). The barcode sequences generated in this study have been deposited in BOLD systems and are available for public use (see Appendix I).

There was a lower interspecific distance between *H. araujoi* and *H. gribodoi* from Kakamega compared to *H. gribodoi* collected from Mwingi and Kakamega, 1.05% and 4.7% respectively. 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 3.1, page 61). The lower genetic distance suggests a possibility of interbreeding between *H. araujoi* and *H. gribodoi* collected from Kakamega. On the other hand, the high genetic distance between *H. gribodoi* from Kakamega and Mwingi (4.7%) suggest a likelihood of cryptic species within *H. gribodoi*. One of the reasons for the higher intraspecific distance is probably an adaptation to the different ecological factors (Cristina et al. 2006). Kakamega forest is located in the highlands of western Kenya and is a tropical rainforest that lies between 1500-1600m above sea level (Tsingalia and Kassily 2009) while Mwingi is a semi-arid mid-altitude (500-800m) area (Kaloi et al. 2005). In addition, stingless bees migrate for short distances, of about fifty to a few hundred meters between conspecific colonies (Roubik 2006). More specific, *Hypotrigona* species mate in an area about 100m around their nests (Portugal-Araujo and Kerr 1959) and they are known to forage for short distances, approximately 300m from their nests (Wille 1983). As such, Mwingi and Kakamega populations are isolated by distance, hence unlikely to mate, which explains the differences observed in the genetic distances.

Chemical profile of the head extracts

In Chapter 4, chemical profiles from the whole head of worker bees were used to separate three *Hypotrigona* species. It was found that *H. gribodoi* and *H. araujoi* are more similar in their chemical profiles compared to *H. ruspolii*. These results provide support for their close evolutionary relationships as depicted by the genetic distances shown in Chapter 3 (Fig 3.1, page 62).

Separation of *Hypotrigona* species was not only achieved based on uniquely missing components but also by the presence or absences of specific components. Eight chemical compounds were specific to *H. araujoi*, six to *H. gribodoi* and nine to *H. ruspolii*. In *H. ruspolii,* four out of nine chemical components contributed to the separation of *Hypotrigona* species, which include heptacosene, *n*-dotriacontane, aldehyde octadecanal and the alcohol 1-eicosanol. However, none of the chemical component specific to *H. gribodoi* contributed to the separation of *Hypotrigona* species. This could be because *H. gribodoi* is a more derived species group of the three (Chapter 3). The specific compound profiles might point to their function as signals in the communication system of stingless bee (Leonhardt et al. 2009).

Recommendations and suggestion for future studies

There is a need for more extensive field sampling in other sites in Kenya and in Africa at large to have a wider coverage of the 20 stingless bee species. This will result to more comprehensive data on nesting sites and nest architecture of African stingless bees. The cryptic species detected in this study should be studied further using nuclear markers such as microsatellite and Internally Transcribed Spacer (ITS) of the ribosomal gene. The markers would show if there is gene flow between populations and if these populations are distinct. The nuclear markers can be combined with geometric morphometrics and used instead of traditional morphometrics. If an extensive study will be carried out, there is possibility of identification of unknown *Hypotrigona* species. In future the *Hypotrigona* species propolis and nest entrance volatiles should be studied for species discrimination. It is also recommended that stingless beekeepers should collect bees in the forest using traps as described in Oliveira et al. (2013) to avoid cutting down tree while harvesting stingless bee colonies. Finally, separation of *Hypotrigona* species has been achieved in this study. This provides a baseline and opens up for further research on their biology and behavior.

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APPENDIX III

Simplified *Hypotrigona* species Key for farmers/ researchers (Chapter 2)

After location of the nest in the field/ meliponiary, the first feature to look out for and observe is the external nest entrance, colour and size of the apical opening;

White or cream and narrow.....*H. gribodoi*

Yellowish-brown and broad.....…….*H. araujoi*

Dark brown….....*H. ruspolii*

Internal nest features for use in meliponiaries

Arranged in semi comb-like vertical layers and presence of strong pillars .....*H. araujoi*

Brood cells clustered.....*H. gribodoi or H. ruspolii*

Brood cells covered, fully or partially, with involucrum.....*H. ruspolii*

Colour of propolis

Reddish brown …….……*H. araujoi*

Dark brown……..*H. ruspolii*

Light brown…….*H. gribodoi*

APPENDIX IV

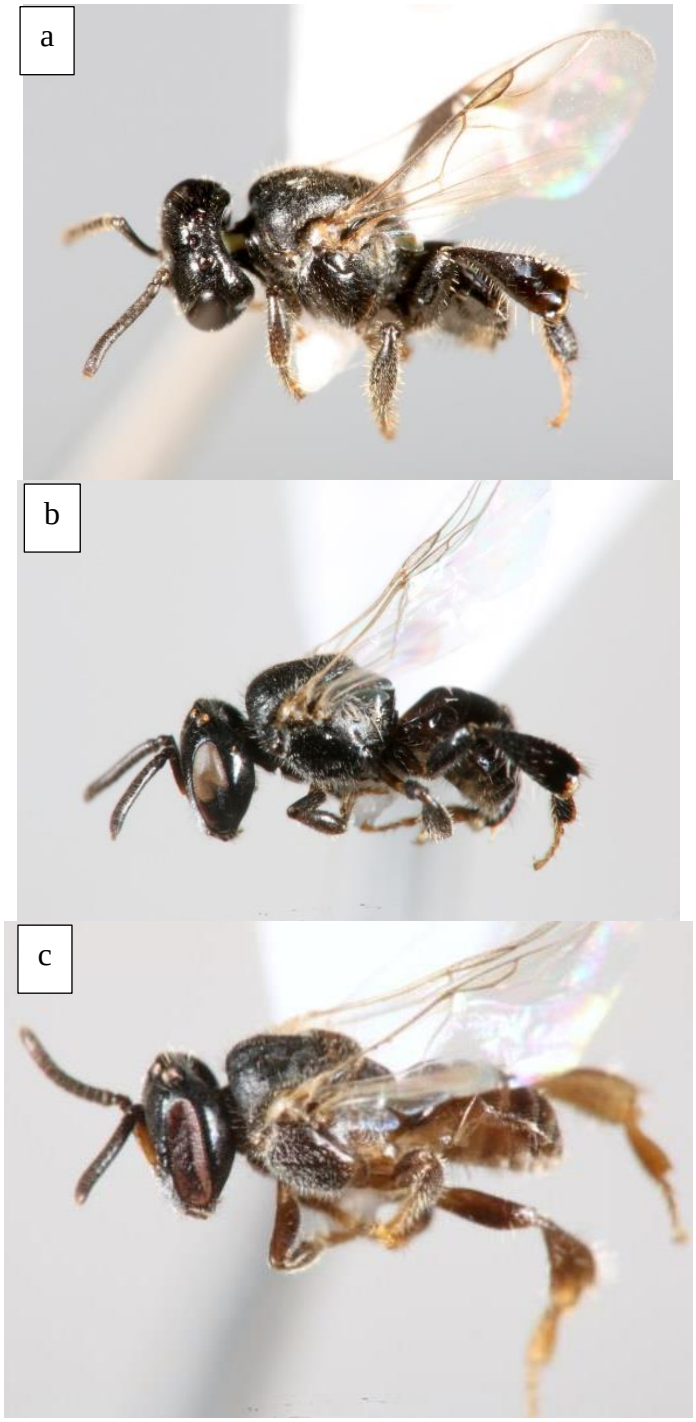

Fig. 1. The three *Hypotrigona* species; a) *H. araujoi*, b) *H. ruspolii* and c) *H. gribodoi*. (Mg=X17)

APPENDIX V

Fig. 1. *Hypotrigona* species fore wings, hind wings and hind legs. Mg=X35