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

N.N. Ndungu[1,2*], A.A. Yusuf [2], S.K. Raina[1], D.K. Masiga[1], C.W.W. Pirk[2] & K. Nkoba[1]

[1]*International Centre of Insect Physiology and Ecology (ICIPE), P.O. Box 30772-00100, Nairobi, Kenya*
[2]*Social Insects Research Group, Department of Zoology and Entomology University of Pretoria, Private Bag X20, Hatfield, Pretoria, 0028 South Africa*

Hypotrigona species are difficult to identify morphologically. Here, we show that nest sites and nest architecture can be used to discriminate 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 of Insect Physiology and Ecology (ICIPE). The following parameters were recorded: nest sites, internal nest entrances, external nest entrance colour and size, sizes (in terms of volume) of brood cells, honey and pollen pots, arrangement of brood cells and presence or absence of involucrum (cerumen covering brood). It was found that nest sites are specific to species. *Hypotrigona gribodoi* nests mostly in crevices in mud walls while *H. ruspolii* and *H. araujoi* nest in cavities in specific tree species, mainly in indigenous forests. The colour of external nest entrances varies between the species. *H. araujoi's* is yellowish brown, *H. gribodoi's* is white or cream while that of *H. ruspolii* is dark brown. 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 *H. araujo*'s form vertical semi comb-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 *Hypotrigona* species. Therefore, nest sites and nest architecture can be used to discriminate three *Hypotrigona* species. Furthermore, the study indicates that conservation of indigenous forests, the main habitat for *H. araujoi* and *H. ruspolii* is important for their conservation.

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

INTRODUCTION

Stingless bees are a group of bees found in tropical regions of the world (Michener 2007; Michener & Grimaldi 1988; Rasmussen, Nieh & Biesmeijer 2010) where they play an important ecological role as pollinators of many wild and cultivated plants (Heard 1999; Nkoba *et al*. 2014; Slaa *et al*. 2006). Stingless bees produce honey that is important for subsistence in many rural communities although they produce less than honey bees (Eardley & Kwapong 2013; Nkoba *et al*. 2012, 2016). Unlike honey bees for which only 11 species have been described in the genus *Apis* (Michener 2007), stingless bee taxa are diverse with over 60 genera in which over 600 species have been reported so far (Michener 2007; Rasmussen & Cameron 2010). Contrary to Neotropical stingless bees where several studies have been done on their taxonomy, biology, ecology and genetics, African species have been less studied and thus the classification of the group is not fully resolved (Eardley 2004; Eardley & Kwapong 2013; Michener 2007). The recent taxonomic revision by Eardley (2004) provides identification keys for all African stingless bees known at the time, based on morphology. Six genera have been identified that comprise 20 species (Eardley 2004) among which 12 species are known to occur in Kenya (Ndungu *et al*. 2017; Nkoba 2012). The six genera include *Dactylurina* Cockerell, *Meliponula* Cockerell, *Plebeina* Moure, *Hypotrigona* Cockerell, *Liotrigona* Moure and *Cleptotrigona* (Sakagami, Roubik & Zucchi, 1993). *Hypotrigona* consists of four species namely, *Hypotrigona gribodoi* (Magretti, 1884), *H. ruspolii* (Magretti, 1898), *H. araujoi* (Michener, 1959) and *H. squamuligera* (Benoist, 1973) (Eardley 2004). *Hypotrigona squamuligera* occurs only in West Africa; while the three other species are present in different habitats in East Africa. *Hypotrigona*

*Author for correspondence. E-mail: nellymucugu@gmail.com

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species are difficult for even taxonomic experts to differentiate due to their very similar body morphology (Eardley 2004; Michener 1990, 2000). In a recent study, the three *Hypotrigona* species were separated using morphometrics and molecular tools, *i.e.* using COI sequences (Ndungu *et al*. 2018a) and using chemical extracts from heads (Ndungu *et al*. 2018b). However, the molecular tools and chemical profiles methods are most readily applied in laboratory environments. Stingless beekeepers and researchers need easy ways of discriminating *Hypotrigona* species in the field and in meliponiaries, based on external and internal features of their nests. The accurate identification of species is required for colony propagation that involves techniques such as queen production and colony division (Slaa *et al*. 2006).

Apart from body morphology, mitochondrial DNA and chemical profiles from head extracts as tools for species differentiation, nest architecture and nesting ecology can also be used to identify stingless bee species. The nest architecture of stingless bees in South America and Australia has been studied (Barbosa *et al*. 2013; Oldroyd & Pratt 2015; Franck *et al*. 2004). However, little has been reported on nest architecture differentiation between *Hypotrigona* species in Africa. Portugal-Araujo & Kerr (1959) and Michener (1959) reported that *H. gribodoi* has clustered brood cells while *H. araujoi* has vertical single-layer combs. 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, a detailed examination of nest architecture in three *Hypotrigona* species found in Kenya, namely *H. gribodoi*, *H. ruspolii* and *H. araujoi*, was carried out in order to develop tools for field identification.

MATERIAL AND METHODS

Nest sampling and species identification

During 2014–2015, nests of *H. gribodoi*, *H. araujoi* and *H. ruspolii* species were collected from two ecological zones in Kenya, namely Kakamega (0°09′N 34°50′E) and Mwingi (0°51′S 38°22′E) (Fig. 1). Random searches for *Hypotrigona* species 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; Kapwong *et al*. 2010). *Hypotrigona araujoi* and *H. ruspolii* nests were mostly located in the Kakamega indigenous forest in pre-existing cavities in living trees. *Hypotrigona araujoi* nests were mainly collected from five tree species namely *Croton silvaticus*, *Prunus africana*, *Funtumia africana*, *Antiaris africana* and *Olea capensis*. On the other hand, *H. ruspolii* nests were taken from six tree species; *Cordia africana*, *Croton silvaticus*, *Prunus africana*, *Funtumia africana*, *Olea capensis* and *Ficus umbellata*. In Mwingi and Kakamega, *Hypotrigona gribodoi* nests were found in crevices of mud house walls in homesteads. The *Hypotrigona* specimens collected in the field were identified using the taxonomic keys of Eardley (2004) and Michener (1959). To separate the *Hypotrigona* species molecular tools were also applied (Ndungu *et al*. 2018a), 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 their natural habitats (Kakamega and Mwingi) and for each colony the brood cells with queen and worker bees were transferred into an ICIPE-1H hive design (26 (l) × 6 (w) × 5 (h)) cm (Kiatoko 2012). In total 55 colonies were collected; 30 *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 allowed to settle in meliponiaries in Kakamega and Mwingi for three weeks and later transferred to a meliponiary stationed at ICIPE in Nairobi, Kenya (1°13′S 36°53′E). At ICIPE, the colonies were allowed to settle for one more week before starting the experiment.

Data collection and analysis

To assess the nest architecture variations between three *Hypotrigona* species, we collected parameters on the shape, colour and surface area of the apex of nest entrances (Kiatoko 2012). The presence or absence of an internal nest entrance structure was also recorded. An internal nest entrance is defined as the extension of the external nest entrance tube into the nest. The brood cells' arrangement, colour and volume were also recorded and photographed (Oldroyd & Pratt 2015; Roubik 2006; Roubik 1983; Michener 1959). The volume (mm^3) of honey and pollen pots size was calculated. The colour of cerumen was determined by reference to the RGB colour system (http://www.rapidtables.com/web/color/RGB_Color.htm#rgb-format).

Furthermore, the presence or absence of an inner involucrum, outer pillars and garbage sites in the nests were also observed and photographed (Barbosa *et al*. 2013; Oldroyd & Pratt 2015; Roubik 2006).

The shape of the opening at the apex of the entrance tube was determined by calculating the ratio (R_2/R_1) from the measurement of the minor opening (R_1) to that of the major opening (R_2). The open entrance tube with a ratio equal to 1 ($R_1 = R_2$) was described as circular and that with ratio >1 ($R_1 \neq R_2$) were described as oval. A digital Vernier caliper (Gimbel Mexicana, S.A. DE C.V, Mexico) was used to take measurements of these axes. The cross section area (mm^2) of the open entrance tube was calculated using the geometric formula for each shape recorded. The surface areas of a circular and oval open entrance tubes were calculated using the formula, $S = \pi \times R2$ and for an oval opening, $S = \pi \times (R1) \times (R2)$ (Couvillon *et al*. 2008). The colour of the entrance and propolis were recorded with reference to the RGB colour system (http://www.rapidtables.com/web/color/RGB_Color.htm#rgb-format).

The arrangement of brood cells was recorded as comb, semi-comb, spiral or cluster (Oldroyd & Pratt 2015). The dimension of the brood cells in terms of volume was estimated from 30 brood cells collected from three hives per species (90 brood cells). The diameters and radii of brood cells were measured under a Zeiss microscope (Germany) equipped with ZEN 2012 imaging software (version 1.1.2.0, Carl Zeiss Microscopy, GmbH) at a magnification of ×0.54. The volumes of brood cells were calculated assuming a spherical shape.

In addition, diameter and radius of isolated honey and pollen pots were measured using digital Vernier calipers. At least 15 honey pots were measured in three colonies of each species (*H. ruspolii* = 67, *H. araujoi* = 41 and *H. gribodoi* collected from Kakamega and Mwingi = 86). The number of pollen pots included in the study were as follows; *H. ruspolii* = 33, *H. araujoi* = 23 and *H. gribodoi* Kakamega and Mwingi = 87. Again, volumes of the honey and pollen pots were calculated assuming a spherical shape, $4/3\,\pi\,r^3$. All photographs in this study were taken using a Nikon Camera Model l830, 34 × wide ED UR, optical 20 cm, 4.0–13.6 mm.

Statistical analysis

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. All these data did not significantly deviate from the normality assumption and homogeneity of variance. Analyses of variance were performed to compare three *Hypotrigona* species on 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, means for the measured parameter were separated using the Tukey HSD test. A *t*-test was used to compare the same parameters for *H. gribodoi* from the two locations of Mwingi and Kakamega to test for variation due to location. α values less than 0.05 were considered to be statistically significant. The statistical analyses were performed using R 3.2.3 (R Core Team 2015).

RESULTS

Nest site and nest entrance architecture

In Kakamega and Mwingi, *H. gribodoi* was found nesting in mud wall crevices, dry tree logs and rocks, while *H. araujoi* and *H. ruspolii* nested in pre-existing cavities in live tree trunks and branches in Kakamega forest. Most nests of *H. gribodoi*, *H. araujoi* and *H. ruspolii* had an external protruding entrance tube (Fig. 1a, d, g). Some *H. gribodoi* nests however did not have a protruding entrance; instead, the bees put soil, pebbles and resin at the entrance (Fig. 1c). The shape, colour and apex surface area (SA) of the outer nest entrance tube varied among the three *Hypotrigona* species (Table 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. 1a, b), reddish brown in *H. araujoi* (Fig. 1e, f) and dark brown in *H. ruspolii* (Fig. 1g, h). It was also observed that for *H. gribodoi* and *H. araujoi*, the sticky droplets occurred at the base of the entrance tube (outer surface where the entrance tube is attached to the substrate). The droplets are mostly laid during the night or when there is a tentative invasion by predators (Fig. 1i).

The shape of nest entrances varied between the three *Hypotrigona* species (Fig. 1). The shape of the nest entrance was circular or oval in *H. gribodoi* (Fig. 1a, b) and oval in both *H. araujoi* and *H ruspolii* (Fig. 1e–i).

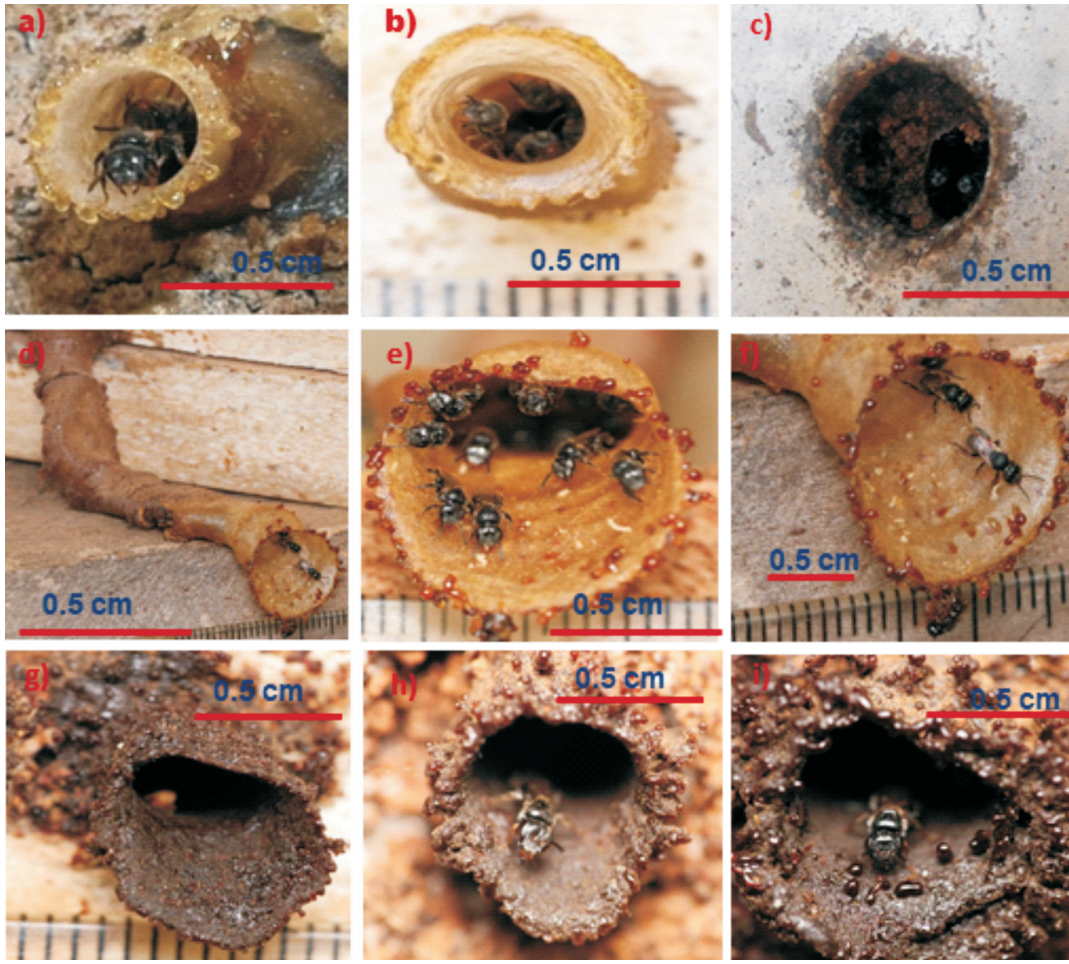

Fig. 1. **a**–**c**, External nest entrances of *Hypotrigona gribodoi*: **a**, circular; **b**, oval with resin droplets at the apex of the nest entrance; **c**, soil pebbles and resin at the entrance (no extruding entrance tube). **d**–**f**, External nest entrance of *H. araujoi*: **d**, circular entrance, also showing long external tube; **e**, resin droplets at the apex of the nest entrance; **f**, oval entrance. **g**–**i**, Oval nest entrances of *H. ruspolii*: **i**, resin droplets at the apex of nest entrance (no extruding entrance tube).

The colour of the nest entrance tubes varied between the three *Hypotrigona* species (Fig. 1). The nest entrance tubes were yellowish-brown in *H. araujoi*, white or cream in *H. gribodoi* and dark brown in *H ruspolii* nests. The mean surface area of the nest entrances at the apex varied significantly between the three species ($F_{2,38}$ = 86.33, P < 0.0001) (Table 1). There were significant differences between means of nest entrance's surface area of *H. gribodoi* (mean 8.42 ± 1.8 mm^2) and *H. araujoi* (128.00 ± 15.1 mm^2, Tukey HSD, P < 0.0001), and between *H. araujoi* and *H. ruspolii* (15.67 ± 2.5 mm$^{2;}$ Tukey HSD P < 0.0001), respectively. There was no difference between *H. ruspolii* and *H. gribodoi* (8.42 ± 1.8 mm^2, Tukey HSD P = 0.69). *T*-test shows that the surface area at the apex of the nest entrance tube for *H. gribodoi* from Mwingi and from Kakamega differed significantly (t_{39} = 8.57, P < 0.0001).

Inside the nest, it was observed that an internal nest entrance tube led to the honey and pollen pots in *H. gribodo*i from both locations (Fig. 2a). Such internal entrances were not observed in *H. araujoi* and *H. ruspolii (*Fig. 2b, c).

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

The brood cells arrangement, colour and sizes

Table 1. Characteristics of the nest entrance of 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
Resin or sticky droplets colour	Reddish-brown	Yellowish-white	Dark brown
Nest entrance surface 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 indicate significant differences. n = number of samples.

varied between the three *Hypotrigona* species (Table 2). In *H. araujoi*, brood cells were arranged in vertical layers. Broods cells in the same layer were attached to each other directly (brood cell to brood cell in direct contact) forming semi comb-like structures (Fig. 3a). The different brood layers were connected by short pillars; the newest brood cells were located on the outermost layers enclosing the older brood cells (as in Fig. 3a). In *H. araujoi* brood cells were yellowish for newly capped cells and yellow brown for cells at the pupal stage (Fig. 3a). The brood cells in a *H. gribodoi* nest were arranged in clusters with short pillars connecting brood cells to each other (Fig. 3b). The newest brood cells were located on top of the older brood cells. The brood cells in *H. gribodoi* were yellow brown for cells at pupal stages and yellow for newly capped cells. Similar to *H. gribodoi*, brood cells in *H. ruspolii* nests were arranged in clusters with short and thin pillars between some brood cells (Fig. 3c). The newest brood cells in *H. ruspolii* were also located on top of the older brood cells. For *H. ruspolii*, the brood cells for newly capped cells (larval brood cells) were metallic brown while at the pupal stage the brood cells were pale.

One unique characteristic in *H. araujoi* nests was the presence of strong and long pillars attached to the top of the brood cells. These protruding pillars were longer (6 cm) and stronger than those between brood cell layers. Such external pillars were absent in nests of *H. gribodoi* and *H. ruspolii* (Fig. 3a–c).

Hypotrigona araujoi had the largest mean volume of brood cells, 12.7 ± 0.1 mm^3 while the smallest volume was recorded in *H. ruspolii*, 6.07 ± 0.1 mm^3, and with *H. gribodoi*'s being intermediate (Table 2). The mean volume of brood cells was also significantly different between the three *Hypotri-*

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

Table 2. Brood cell arrangement, colour and volume (mean ± S.E.) of worker brood cells, in three *Hypotrigona* species.

Characteristic	*Hypotrigona* species		
	H. araujoi (n = 90)	*H. gribodoi* (n = 90, per location)	*H. ruspolii* (n = 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; $t_{212.08} = 0.291$, $P = 0.771$

Different letters in a row or column indicate significant differences. n = number of samples.

gona species ($F_{2,381} = 807.4$, $P < 0.00001$). Pairwise comparisons using a Tukey HSD test showed significant differences in the brood cell volumes between all the three pairs of *Hypotrigona* species ($P < 0.0001$). Analyses using a *t*-test also showed that there were no significant difference between the means volume of brood cells of *H. gribodoi* collected from Mwingi and Kakamega ($t_{212.08} = 0.291$, $P = 0.771$) (Table 2).

Honey and pollen pots size

Honey and pollen pots were mostly clustered, although some pots were scattered in the nests in all the *Hypotrigona* species' nest (Fig. 4a, b, c). Honey and pollen pots were spherical in shape. The mean volume of honey pots and pollen pots varied significantly among the three *Hypotrigona* species ($F_{2,134} = 128.9$, $P < 0.0001$) and ($F_{2,107} = 42.58$, $P < 0.0001$), respectively (Table 3). Tukey HSD showed significant difference between honey pots and pollen pots of these three species ($P < 0.0001$). There was also a significant difference in the volumes of honey and pollen pots of *H. gribodoi* collected from Kakamega and

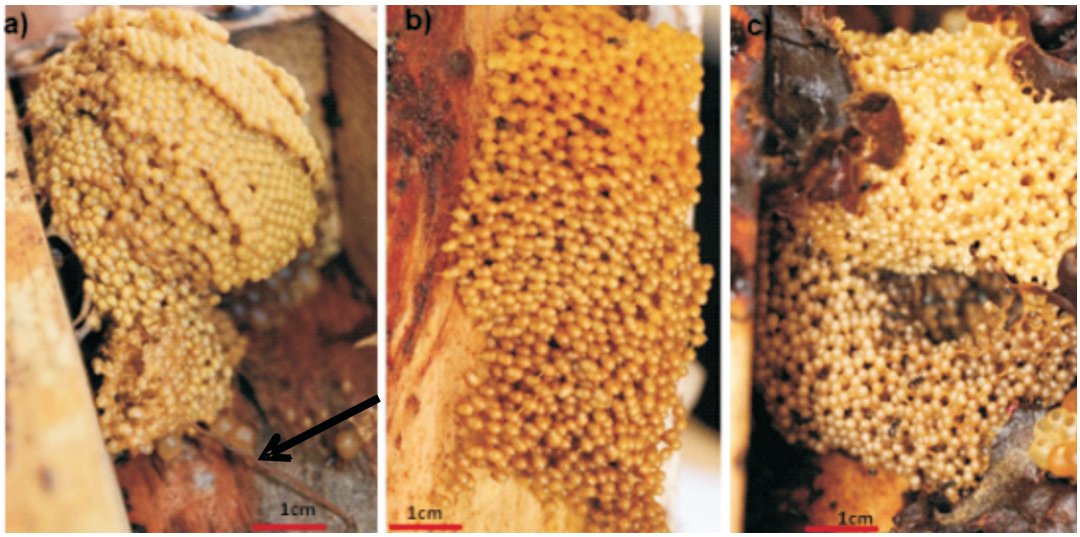

Fig. 3. Arrangement of brood cells in the *Hypotrigona* species. **a**, *H. araujoi* brood cells with old and new brood cells arranged to form semi comb-layers, new brood cells are on the outer layer. Strong pillars are also observed on the brood cells. **b**, Clustered brood cells arrangement as observed in *H. gribodoi*'s nest. The new brood cells are on top of old brood cells. **c**, Clustered arrangement of brood cells in *H. ruspolii* nest. New brood cells are constructed on top of the old ones.

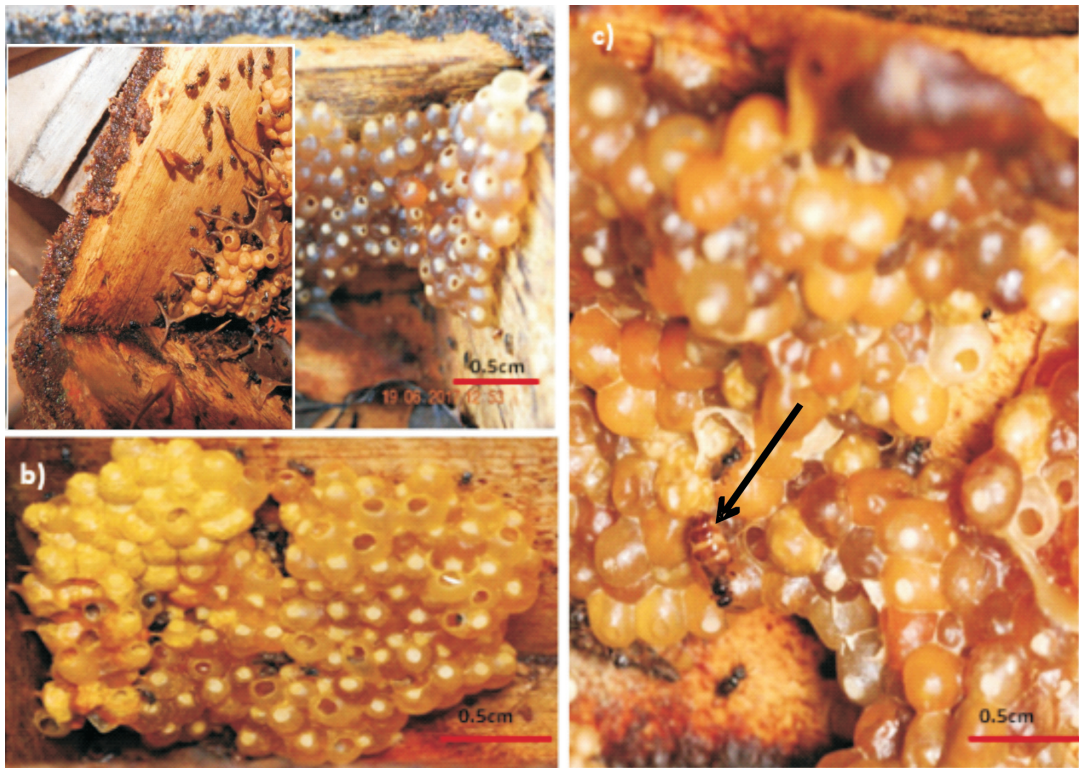

Fig. 4. *Hypotrigona* honey and pollen pots. **a**, *H. araujoi;* **b**, *H. gribodoi*; **c**, *H. ruspolii*. The black arrow points to the queen.

Mwingi (t_{78} = 20.631, $P < 0.0001$; t_{90} = 28.3, $P < 0.0001$).

The involucrum and colour of propolis

In contrast to *H. araujoi* and *H. gribodoi*, one unique characteristic in *H. ruspolii* nest is that brood cells, honey and pollen pots were covered by a dark brown inner involucrum (Fig. 5a, b, c). The colour of propolis used to seal cracks in hives was specific, being reddish brown in *H. araujoi*, light brown in *H. gribodoi*, and dark brown in *H. ruspolii* (Fig. 5d, e, f; refer to Table 3).

Table 3. Involucra, propolis colour and volume (mm^3) 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.

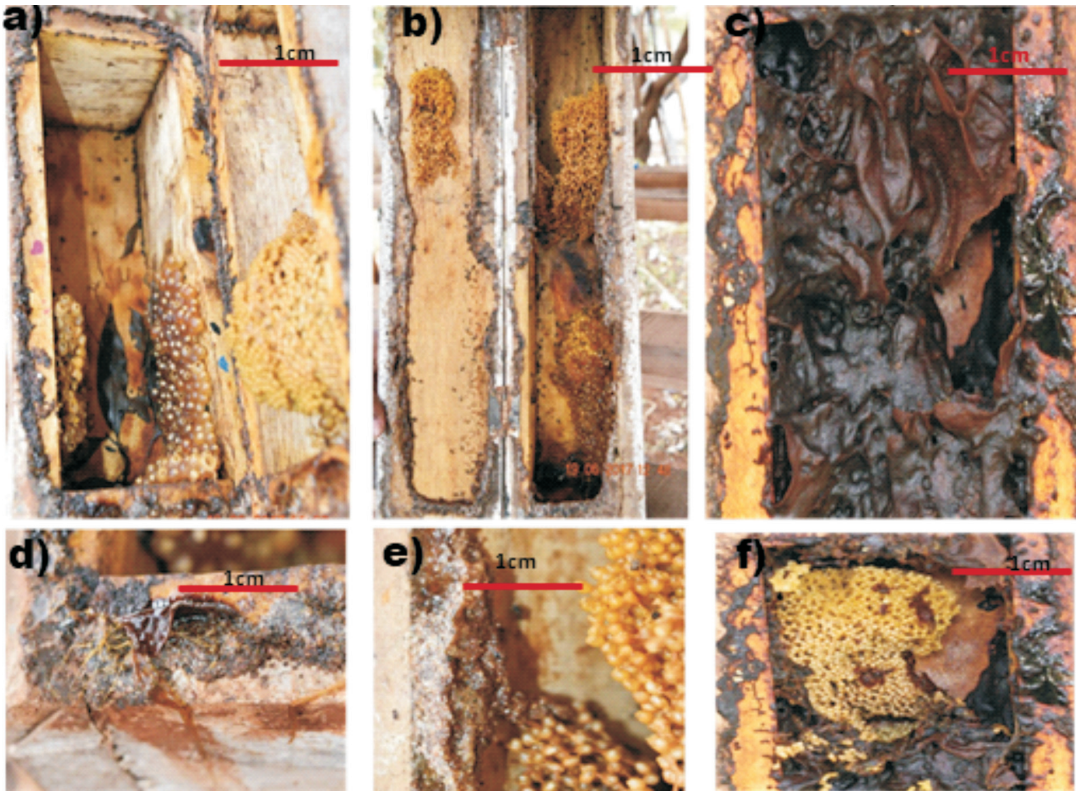

Fig. 5. **a**–**c**, Overview of *Hypotrigona* nests: **a**, *H. gribodoi*; **b**, *H. araujoi;* **c**, *H. ruspolii* – Intact involucrum covering brood cells, honey and pollen pots (indicated by the red arrow). **d**–**f**, Propolis of the *Hypotrigona* species: **d**, *H. araujoi* – reddish brown;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

Garbage site in the nest

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 floor of the hive using sticky propolis. In *H. ruspolii* nests, garbage sites are emptied by workers using their mandibles to transport garbage to the external entrance, which is then dumped immediately outside the nest, whilst in *H. gribodoi* and *H. araujoi*, workers remove garbage differently by flying away with the waste.

DISCUSSION

The general nest architecture in the three *Hypotrigona* species, *H. gribodoi*, *H. araujoi* and *H. ruspolii*, consists of four main compartments as follows: external nest entrance tube; old and new brood cells; honey and pollen pots and garbage-dumping site. This study gives details of key differences in nest architecture including nest entrance structure, brood cell arrangement and size, honey and pollen pot structure and size and propolis and involucrum structure that can be used as identification tools for these three *Hypotrigona* species. According to Eardley (2004), *Hypotrigona* species are the most difficult to identify using morphological features. Stingless bee nest sites and nest architecture have been shown to be specific and can be used for species identification (Rasmussen & Camargo 2008; Roubik 2006). In this study, it was observed that *Hypotrigona* species nest in different habitat types. In Kakamega and Mwingi, *H. gribodoi* nested in mud walls crevices, dry tree logs and rocks, while *H. araujoi* and *H. ruspolii* nest in pre-existing hollows in

trunks and branches of trees found in Kakamega forest. The dry climatic conditions in Mwingi could be a limiting factor for habitation by *H. araujoi* and *H. ruspolii*.

The external nest entrance tube colour in domesticated colonies of *Hypotrigona* species used in this study 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 specific colour of the entrance appeared to be independent of the environmental conditions. Inherent factors like mixing of wax and resin play an important role in creating the specific colour of the entrance tubes. The colour of propolis used for sealing cracks varied between the *Hypotrigona* species; dark brown in *H. ruspolii* nest, reddish brown in *H. araujoi* nest and yellowish white in *H. gribodoi* nest. We suggest that the variation in colour in different species could be as a result of the specific plant resins that each bee species forages for the construction of their nest entrances (Leonhardt 2010; Roubik 2006). Therefore, the colour of the entrance can be used to identify *Hypotrigona* species. In addition, the differences in nest entrances observed in the three *Hypotrigona* species provide a viable character for field identification. Nest entrances in *H. gribodoi* are much narrower than those of the other species described here, while those of *H. araujoi* are wider than the other *Hypotrigona* species. Nest entrance architecture is important for the bees because it allows access for foragers and at the same time assists the guard bees standing at the entrance to exclude intruders (Grüter, Kärcher, & Ratnieks 2010). Narrow entrances are said to keep away intruders, while a wide entrance favours foraging as it allows bees to leave and enter the hive easily (Biesmeijer, Slaa & Koedam 2007; Roubik 2006). Only one or two guard bees occur at nest entrances in *H. gribodoi* and *H. ruspolii*. Furthermore, fresh sticky resin droplets around the apex of nest entrance tubes trap crawling invaders, like ants, which were frequently observed in the field and are reported by Roubik (2006). In *H. araujoi*, the nest entrance is wider than that of the other two species, and to better protect the nest a lot of resin was deposited around the tip and at the outer surface where the entrance tube is attached to the substrate. Six to eight highly aggressive guards occurred at these nest entrances (pers. obs.). According to Roubik (2006), small size bees with wide entrances are found to be highly defensive; Michener (1959) showed that *H. araujoi* was more aggressive than *H. gribodoi*, which is in line with our results on the size of the entrances.

A unique characteristic that was observed in *H. gribodoi* colonies is that the outer entrance leads to an internal entrance that ends near the storage pots. This result is in agreement with previous findings where internal entrance was observed in two colonies of *H. gribodoi* nests in Ghana (Bassindale 1955). We suggest that the internal nest entrance could be used to lead the foragers to the storage pots while intruders may be disoriented and thus leave the nests.

The worker brood cells in *H. gribodoi* and *H. ruspolii* form a cluster type arrangement. This is a characteristic of primitive bees (Kerr & Maule 1964; Wille 1964). However, a specific characteristic was observed in *H araujoi* where brood cells are arranged in vertical layers of semi comb-like structures and thus could be more advanced in terms of evolution compared to *H. gribodoi* and *H. ruspolii* (Ndungu *et al*. 2018a). In addition, *H. araujoi* was the only species that had strong pillars emerging at the top of the brood mainly for attachment to the roof of the nest (Fig. 3).

None of the three species had inner involucra covering the brood cells as is present in most genera of African stingless bees (Barbosa *et al*. 2013). However, dark brown outer involucrum covering the brood cells, honey and pollen pots was observed in *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 control and as defence against small predators, parasites and pathogens (Barbosa *et al*. 2013; Figueiredo-Mecca, Bego & Nascimento 2013; Rasmussen & Camargo 2008). The presence of external involucra is a specific characteristic that can be used to identify *H. ruspolii* bees in the field. An involucrum either surrounds the brood cells (brood cells involucrum), or surrounds both brood cells and storage pots (external involucrum), and is characteristic of primitive stingless bees (Rasmussen & Camargo 2008). Thus, based on the presence of an involu-

crum, *H. ruspolii* is more primitive than *H. gribodoi* and *H. araujoi*. This is supported by molecular data (Ndungu *et al*. 2018a).

In conclusion, nesting sites, nest entrance architecture, brood cells arrangement and the size of storage pot differ significantly between *H. gribodoi*, *H. araujoi* and *H. ruspolii*. Therefore, the tools described in this study can be used to identify these three *Hypotrigona* species in the field. Identifying stingless bees in the field allows tailoring the conservation efforts which is important since *H. araujoi* and *H. ruspolii* nest in cavities in living trees in the forest and are vulnerable to deforestation.

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Supplementary material to:

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African Entomology **27**(1): 25–35 (2019).

SIMPLIFIED *HYPOTRIGONA* SPECIES KEY FOR FARMERS/RESEARCHERS

After location of the nest in the field/meliponiary, the first feature to observe is the external nest entrance, colour and size of the apical opening (Fig. 1 in the Research Article);

Nest entrance white or cream and narrow ..... *H. gribodoi*
Nest entrance yellowish-brown and broad ..... *H. araujoi*
Nest entrance dark brown ..... *H. ruspolii*

Internal nest features for use in meliponiaries (Fig. 3 in the Research Article)

Brood cells 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 (a sheet of a mixture of propolis and resin) ..... *H. ruspolii*

Colour of propolis (Fig. 5 in the Research Article)

Colour of propolis reddish brown ..... *H. araujoi*
Colour of propolis dark brown ..... *H. ruspolii*
Colour of propolis light brown ..... *H. gribodoi*

Vertical non-compartmented hive – ICIPE IHg for the genus *Hypotrigona* (Nkoba 2012).