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

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Abstract

Stingless bees are important pollinators of plants, and also producers of honey. Species within the African stingless bee genus *Hypotrigona* are difficult to 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 species using their chemical profiles. This study shows that head extracts can be used as a reliable taxonomic tool for identifying and differentiating *Hypotrigona* species.

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

Introduction

Stingless bees (Apidae; Meliponini) are important 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). 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 pheromones or kairomones that are species-specific (Gracioli-Vitti et al. 2012) inducing 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, and aggression of the species (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), and these vary with life phases and between castes (Gracioli-Vitti et al. 2012).

Cephalic secretions within species (Lopez et al. 2002) and between closely related species (Francke et al. 2000) show similar chemical profiles. However, those from different species differ as reported from 11 Brazilian social stingless bee species in the genera *Tetragonisca* and *Frieseomelitta* (López et al. 2002). 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 mates and non-nest mates (Francke et al. 2000).

Chemical compositions of the mandibular 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 (López et al. 2002; Patricio et al. 2003; Cruz-López et al. 2005). By contrast, little is known on the cephalic secretions from African stingless bees (reviewed in 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 genus *Hypotrigona* consists of four species; *H. gribodoi, H. araujoi, H. ruspolii* and *H. penna* 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 whole body length. It was further confirmed through breeding experiments by Michener (1959) that *H. araujoi* and *H. gribodoi* could not mate and were indeed two separate biological species. In addition, Eardley (2004) used morphologies of the legs, wings, heads and thoraces 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 three *Hypotrigona* species presently identified in Kenya. Secondly, 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. 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. 1 Map of Kenya showing the two sampling areas, Kakamega forest (green) and Mwingi (chequered box). (modified from Ndungu et al. (2018)

Head extracts: Bees were immobilised on ice, decapitated and their heads were placed into clean pre-labelled 2ml sample vials containing 200µl of HPLC grade dichloromethane. Samples were then 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 taken 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 based on 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 on a GC. While addition of an internal standard allows for quantification of the individual chemical components relative to the mass ratios (RMR) and peak areas of the internal standards.

Gas chromatographic – mass spectrometric analysis (GC-MS): 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 allows for all the components as well as 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 spectra were recorded between 40–600 m/z at 70 eV with a scan speed of 2500. Compounds were identified based on 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 (see supplementary material 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 was purchased from ChemTik, Germany, while all 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: 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 involves 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 were 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 by 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. 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

A total of 50 compounds were identified, 48 of which belong to six chemical classes. These comprise of hydrocarbons of chain lengths C18 to C34, mainly alkanes and alkenes; monohydric, mono- and polyunsaturated aliphatic alcohols, an aldehyde; saturated and unsaturated (ω-3 to ω-9) fatty acids; mono and sesquiterpenes, steroids and two unidentified compounds (Table 1 and Fig. 2).

Qualitative chemical profiles from the head extracts of *Hypotrigona* species and species specific compounds

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 1). While those of *H. ruspolii* contained only four classes including an aldehyde with no terpenoids and no unidentified compounds (Table 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%); hydrocarbons (24.50 ± 3.00% and 27.22 ± 4.20%) and minor proportions of terpenoids. The major chemical compounds found in the three *Hypotrigona* species, *H. araujoi, H. gribodoi* and *H. ruspolii* included; *n*-tricosane, *n*-heptacosane and octadecanoic acid, respectively (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* respectively (Table 1).

The most abundant compounds in the profiles of *H. araujoi* were octadecanoic acid, *n*-tricosane and octadecanol (Fig. 2). In *H. gribodoi*: E-octadec-9-enoic acid, Z,Z octadeca, 9,12-dienoic acid, octadecanoic acid and octadecanol. By contrast, profiles of *H. ruspolii* were dominated by *n*-nonacosane, *n*-tricosane and 1-nonacosanol (Fig. 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 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 1) were unique to the profile of *H. ruspolii*.

Table 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***
		#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. 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.

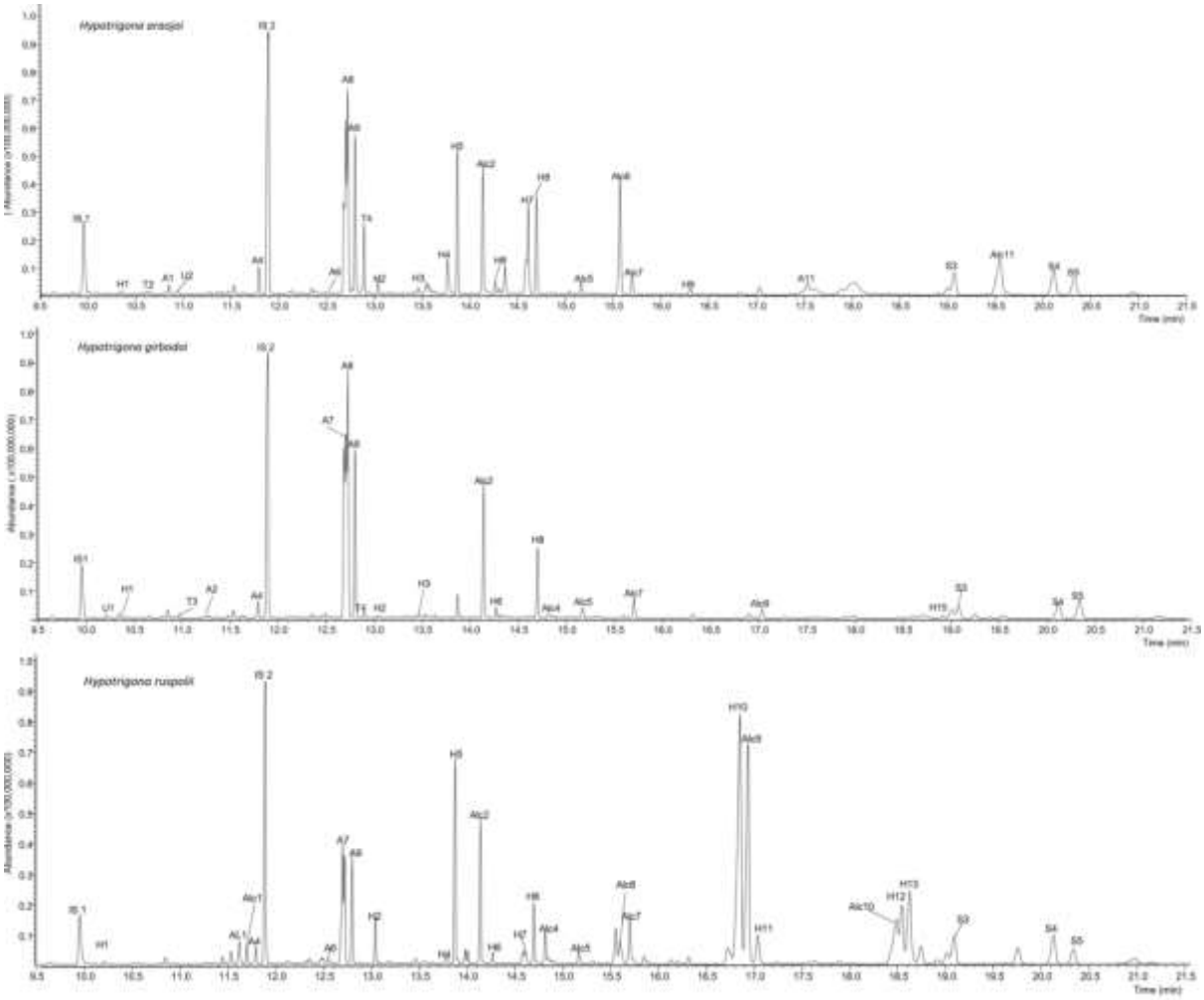

Fig. 2 Representative total ion chromatograms (TICs) of the chemical profiles from head extracts of *Hypotrigona araujoi, H. girbodoi* and *H. ruspolii* workers respectively. IS1 and IS 2 are the internal standards heptadecane and hexadecanoic acid. Compound were identified by their IDs as listed in Table 1 where A = fatty acids, AL = aldehyde, Alc = alcohols, H = hydrocarbons, S = steriods, T = terpeniods and U = unidentified compounds

Quantitative chemical profiles from head extracts of *Hypotrigona* species

Quantitatively, 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 in the three *Hypotrigona* species (ANOVA, Wilks' λ = 0.05, F(16,22) = 4.7744, df =18, P =

0.00045) (Fig. 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 significant 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. 3). Using n-MDS with Bray-Curtis dissimilarity, workers of the *Hypotrigona* species were successfully grouped into their respective species (Fig. 4).

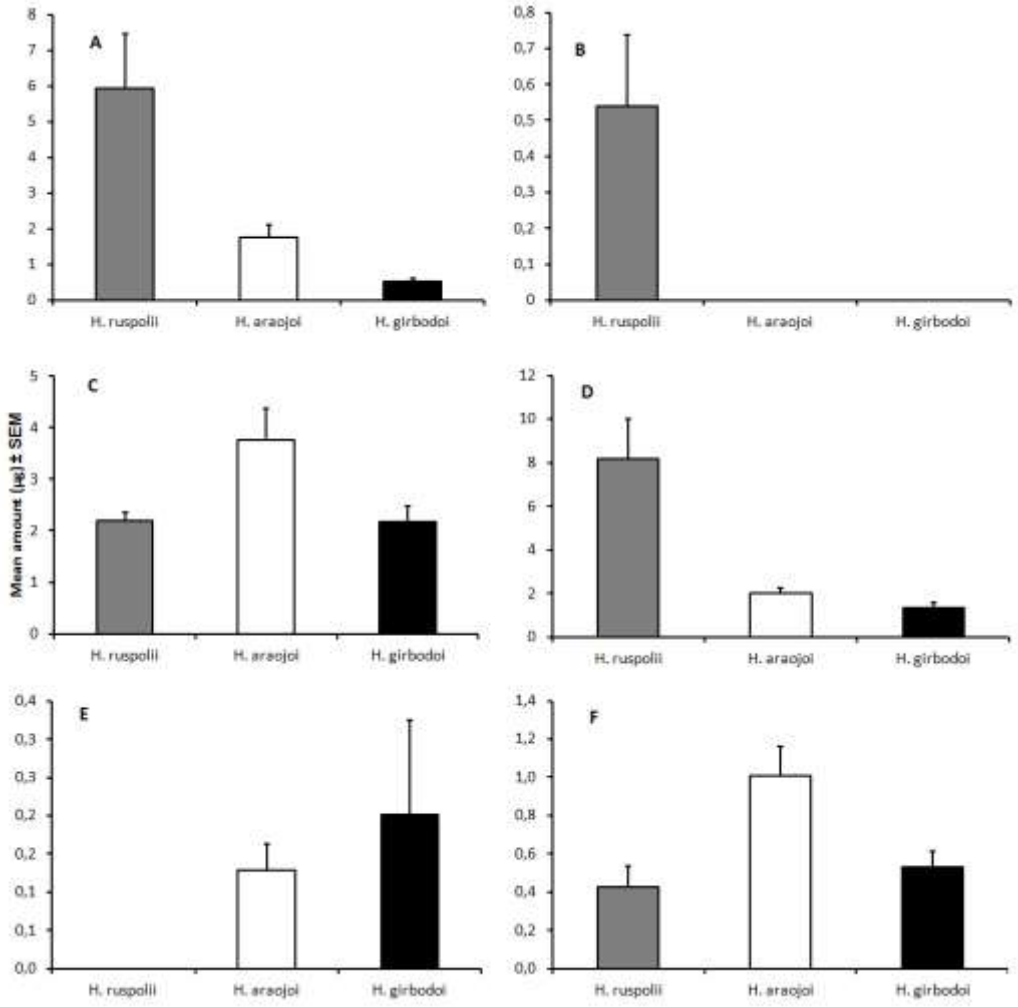

Fig. 3 Concentration (µg) (mean ± SEM) of the classes of compounds identified from head extracts of *Hypotrigona ruspolii* (grey bars), *H. araujoi* (open bars) and *H. gribodoi* (black bars). A = alcohol, B = aldehydes, C = fatty acids, D = hydrocarbons, E = terpenoids and F = steroids. Note the difference in the scale for the Y axis

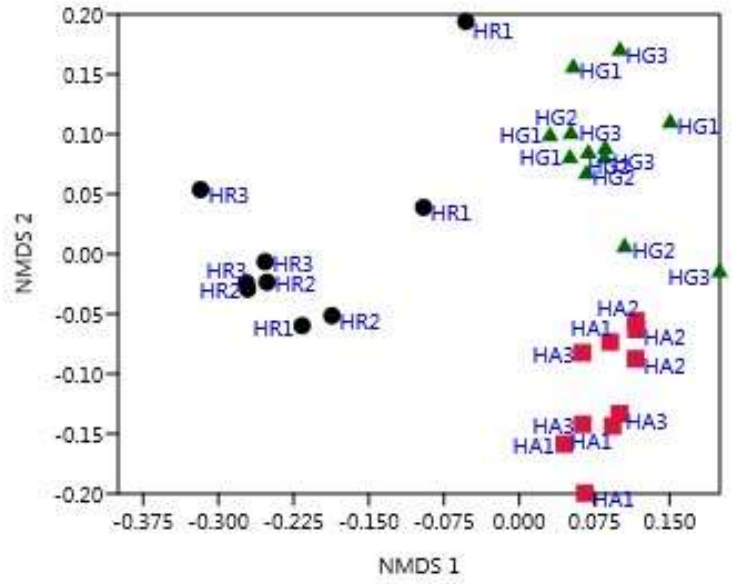

Fig. 4 N-MDS plot of components 1 and 2 showing the separation of *H gribodoi, H. araujoi, H. ruspolii* into their respective species. The species are represented by the first two alphabets followed by colony numbers. HR=*H. ruspolii*, HA=*H. araujoi* and HG=*H. gribodoi*

Discussion

We found differences in the chemical profiles of head extracts from the three *Hypotrigona* species. A total of 50 compounds, 48 from six chemical classes and two unknowns were identified, with the profile of *H. ruspolii* containing 33 compounds, whilst those of *H. araujoi* and *H. gribodoi*, contained 29 and 26 respectively. Extracts from whole heads contain chemical compounds of both surface (cuticular) and glandular origin including those of mandibular and labial glands secretions often used in social insects communication (Free 1987; Leonhardt 2017). Using whole head extracts for chemotaxonomic studies is easier in comparison to glandular dissections since the procedure 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 *Frieseomellita* species were found to have roughly the same composition of cephalic secretions (López et al. 2002).

In this study, six classes of compounds; hydrocarbons, alcohols, fatty acids, terpenes, steroids and aldehydes were identified. For the hydrocarbons, alkanes and alkenes were the two major classes 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 guards bees; or changes in temperature and humidity while alkenes have been shown to be involved in communication in the honey bee *Apis mellifera* (Dani et al. 2005). In addition, cuticular hydrocarbons have been reported to be used for communication by 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.

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

We found high levels of alcohols in all species. Some alcohols such as 1-tetracosanol and tricosanol were common to all species, whilst others were species 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. For example, 2-heptanol had been reported mainly in Brazillian stingless bee (Francke et al. 2000) whilst, 2-heptanol, 2-octanol, Z-5-tetradecenylbutanoate and Z-7-hexadecenylbutanoate were found in *S. postica* (Engels et al. 1997). In addition, 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* species, suggesting it could have been acquired after the splitting of the African –Australian and neotropical clade of stingless bees (Rasmussen and Cameron 2010).

In this study, minor quantities of terpenoids were found 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 resins which are known to contain terpenes and are used for nest construction and defense (Leonhardt et al. 2009). Here, geranyl linalool was common to two species *H. gribodoi* and *H. araujoi* whilst isoborneol and farnesol were species specific and present only in *H. gribodoi* and *H. araujoi* respectively.

Our study on *Hypotrigona* shows that chemical components can be effectively used in the taxonomic separation of the three species (see Fig 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 quantity and quality. The chemical profiles are species specific and therefore could be utilised in the identification of African *Hypotrigona* genus up to species levels.

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Supplementary material

Table S1 Compounds from head extracts of *Hypotrigona* species and the methods used to identify each by 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. 1, MS = published mass spectra from MS libraries (NIST and Wiley), RI = retention index and ST = identification confirmed using synthetic standard compound.