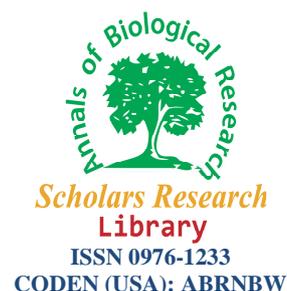




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Geometric morphometrics reveals morphological differentiation within four African stingless bee species

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ABSTRACT

Geometric morphometric techniques were used to assess possible ecotypes within four species of African stingless bees from three different ecological zones in Ghana. Canonical Variate Analyses, Discriminant Analyses, Principal Component Analyses among other techniques evaluated shape variations of eight homologous landmarks on right forewings and effectively discriminated 16 possible ecotypes among 39 populations of stingless bees. It is therefore recommended to stakeholders in meliponiculture to consider the results of this study when exploring movement of colonies from one ecological zone to another.

Key words: Genetics, wings, *Meliponula*, *Dactylurina*, *Hypotrigona*, *Liotrigona*, *Axestotrigona*, ecozones, Ghana ecotypes

INTRODUCTION

Several studies have revealed strong relationship between an organism's morphological traits and its ecological requirements (1-5). According to Baltanás (6), 'the amount of morphospace of an organism is reported to be a good indicator of its ecological diversity (7), evolutionary radiation (8), morphological convergence in distinct communities (9) or selective extinctions (10).

The African stingless bees, like most of its sister groups on other continents are known to play key roles by providing essential ecosystem services such as pollination of both natural vegetation and commercial crops. They provide hive products which are nutritious and medicinal (11-15) and are also of great commercial interest for local people (16). The environments that serve as refugia for bees can lead to speciation among many bee taxa; and

subsequently increase bee biodiversity. However, traits such as behavioral variations among species are very difficult to distinguish; hence most taxonomic studies are based on measurable morphological traits (17-18).

Currently, over 30 African stingless bee species are found within six genera on the continent and more species are yet to be identified. Five of these species have been successfully cultured at the International Stingless Bee Centre (ISBC) in Ghana for honey production and pollination services (19). The six African genera include *Meliponula*, *Dactylurina*, *Hypotrigona*, *Liotrigona*, *Plebeina* and *Cleptotrigona*. Most bees within these genera are flower visitors of many plants except bees within genus *Cleptotrigona*, which are known to be parasitic (20-22).

These genera were classified based on mainly traditional taxonomy which relies on basic morphological characters such as nature of wing venation (reduced number of submarginal cells, visibility of vein Rs), body length, integumental maculations, modifications on hind tibia (presence or absence of corbicula, density of hair and angles at which leg is held), size of first metasomal segment, etc. (23). The phylogeny of the African stingless bees have been dealt with in various publications (24-26), with the earliest being Willie's work (27). Many studies today have successfully separated the Africa genera using molecular studies (28-32).

With the growing pressure on the environment, and the associated loss of honey bees, there exist the need for additional pollinator species to be used in agriculture to maintain resilience in food production and improvement in yield. This, the stingless bees have proved to be a better alternative (15, 19, 23). However, traditional methods of identification for large data set are often expensive, cumbersome and require some level of taxonomic training. It is therefore necessary to assess different modern techniques that are less expensive, much simpler to apply and have successfully been used to discriminate bee species globally. Presently, geometric morphometric analyses of bee wing have provided effective and efficient means of identification among various bee taxa (5, 33-39).

In this study, geometric morphometric techniques are used to discriminate populations of four species of Africa stingless bees (*Axestotrigona ferruginea*, *Meliponula bocandei*, *Dactylurina staudingeri* and *Hypotrigona gribodoi*) from three different ecological zones in Ghana. This research aims at evaluating the possible existence of ecotypes within each of the four species under study, in order to assist in their management and conservation.

MATERIALS AND METHODS

Specimen Collection and Identification

One thousand, nine hundred and sixty (1,960) specimens from four species of African stingless bees (*Axestotrigona ferruginea*, *Meliponula bocandei*, *Dactylurina staudingeri* and *Hypotrigona gribodoi*) were collected from 40 colonies within three ecological zones (Deciduous Forest, Guinea savanna and Transition Zone) in Ghana (Table 1). Each ecozone was subdivided into smaller sets according to the geographical position within that particular ecozone for easy assessment. For instance, Deciduous forest was subdivided into Deciduous Central (DC) (bees from Bobiri Forest Reserve (Bo)), Deciduous South (DS) (populations from Assin Akrofuom (Aa), Assin Fosu (Af), Abrafo (Abr) and Mfuom (Mf)) and North-East of Deciduous forest (NED) (populations from Abotoase (Ab), Pampamwie (Pa) and Kuro (Ko)). The Guinean savanna was subdivided into Guinea North (GN) and Guinea South (GS). GN consist of populations from Jirapa (Ji), Lawra (La), Hamile (Ha), Nadoli (Na) and Wa (Wa), while GS constituted populations from Kyabobo National Park (Ky) (Figure 1). Specimens of *A. ferruginea* and *M. bocandei* were successfully collected from all ecozones, however specimens of *Dactylurina staudingeri* and *Hypotrigona gribodoi* used in this study were obtained from only Deciduous Forest and Transition Zone.

Characteristics of the three Ecological Zones

Deciduous Forest

Currently, the total land area of this zone is unknown; however, FAO-RAF in 2000/1 estimated that Ghana's deciduous forest was about 66300 km² in extent and formed about 90 % of the total forest zone. Bimodal rainfall distribution pattern characterizes this zone and the mean annual rainfall ranges from 1400 to 1750 mm. The mean annual maximum temperature varies from 28.7 to 31°C with a corresponding minimum temperature of 20.6 to 23.2°C. Potential evapotranspiration in this zone is about 1350 mm/yr. The indicator trees are characterized by *Antiaris africana* and *Chlorophora excelsa*. It is within this zone that most food crops and cocoa cultivation takes place. Most of the timber for both local needs and export comes from this zone. As a result of these activities the vegetation outside forest reserves consists mainly of forb regrowth, thicket, secondary forest and swamp thicket ((Figure 1) 40).

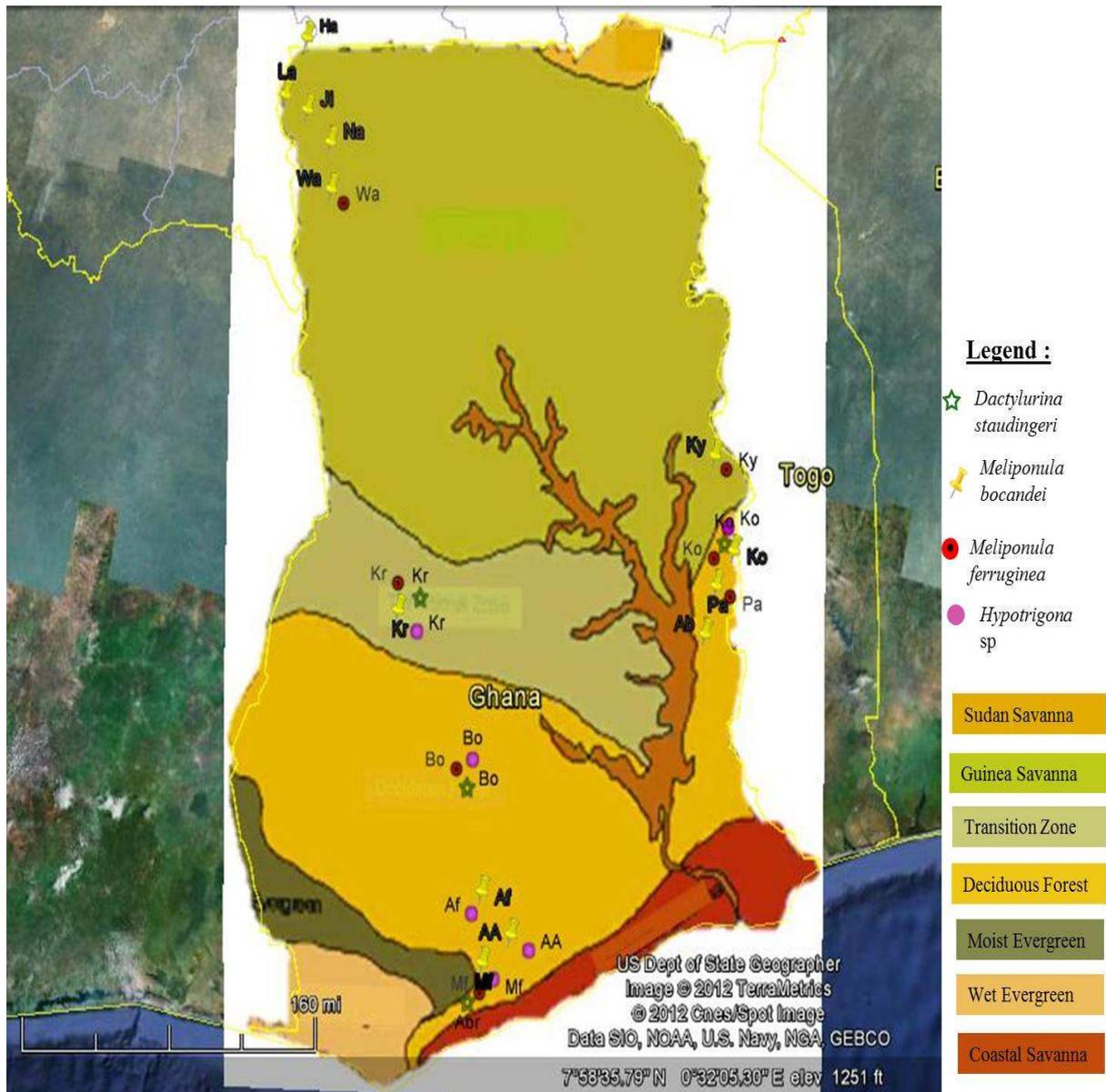


Figure 1. Map of Ghana showing the ecozones and the data collection sites

Guinea savanna

The guinea savanna zone which covers almost the northern two-thirds of the country is the largest ecological zone. Its aerial extent is about 147900 km² characterized by a unimodal rainfall regime lasting from April to October with a mean annual rainfall varying between 1000 and 1200 mm. Monthly total increases gradually from March until a maximum is reached in August or September, after which monthly total falls rapidly. It is dry from November to March. This is the period when the desiccating effect of the harmattan is strongly felt. Mean annual maximum temperature ranges from 33 °C to 35 °C with a minimum of about 22 °C and a mean of 27.8 to 28.5 °C. Relative humidity is about 40 % in the dry season but may reach 84 % during the peak of the rainy season in August. Potential evapotranspiration varies between 2000 and 2300 mm/year (40). The vegetation consists typically of a ground cover of grasses of varying heights interspersed with generally fire resistant, deciduous, broad leaved and gnarled trees at the forest margins in the south. Fringe forest and woodland may be found along the water courses (40-41).

Table 1. Number of stingless bees, sampled localities and coordinates within ecological zones

Locality/Colony Code	Number of bees	Elev.(ft)	Longitude (W)	Latitude (N)	Ecological Zone
<i>Meiponula bocandei</i>					
Kankram, Brong Ahafo-KrB	52	394	001°57'585"	07°36'306"	Transition zone
Pampawie, Volta-PA1B	25	287	000°20'287"	07°48'951"	Deciduous Forest
Pampawie, Volta-PA2B	66	287	000°30'287"	07°48'951"	Deciduous Forest
Kyabobo National Park, Volta-KY3B	41	776	000°28'549"	08°20'406"	Guinea Savanna
Abotoase, Volta-ABB	24	418	000°20'701"	07°25'039"	Deciduous Forest
Abotoase, Volta-AB1B	27	418	000°20'701"	07°26'039"	Deciduous Forest
Abotoase, Volta-AB2B	54	418	000°20'701"	07°25'039"	Deciduous Forest
Koro, Volta-KoB	23	287	000°32'287"	07°54'133"	Deciduous Forest
Koro, Volta-Ko3B	17	287	000°28'287"	07°56'133"	Deciduous Forest
Koro, Volta-Ko4B	36	287	000°28'287"	07°56'133"	Deciduous Forest
Koro, Volta-Ko5B	25	287	000°18'287"	07°24'951"	Deciduous Forest
Mfonom, Central-MfB	40	131	001°24'260"	05°22'595"	Deciduous Forest
Assin Akrofuom, Central-AAB	23	140	001°35'227"	05°12'196"	Deciduous Forest
Assin Foso, Central-AfB	36	148	001°45'188"	05°20'511"	Deciduous Forest
Nadoll, Upper West-NAB	28	910	002°31'520"	10°20'266"	Guinea Savanna
Jirapa, Upper West-JIB	22	1007	002°42'000"	10°32'000"	Guinea Savanna
Hamle, Upper West-HAB	25	897	002°45'019"	10°59'548"	Guinea Savanna
Wa, Upper West-WAB	14	1075	002°30'018"	10°03'540"	Guinea Savanna
Lawra, Upper West-LAB	25	974	002°52'595"	10°38'547"	Guinea Savanna
<i>Axestotrigona ferruginea</i>					
Kankram-Brong Ahafo-KrF	60	341	001°54'321"	07°38'041"	Transition zone
Pampawie, Volta-PAF	126	287	000°30'287"	07°48'951"	Deciduous Forest
Kyabobo National Park, Volta-KYF	21	857	000°33'188"	08°19'302"	Guinea Savanna
Bobiri Forest Reserve, Ashanti-Bo1F	103	757	001°22'436"	06°43'404"	Deciduous Forest
Bobiri Forest Reserve, Ashanti-Bo2F	35	757	001°22'436"	06°43'404"	Deciduous Forest
Koro, Volta-KoF	9	287	000°18'287"	07°24'951"	Deciduous Forest
Koro, Volta-Ko6F	16	287	000°18'287"	07°24'951"	Deciduous Forest
Koro, Volta-Ko7F	18	287	000°18'287"	07°24'951"	Deciduous Forest
Mfonom, Central-MfF	16	192	001°23'216"	05°21'092"	Deciduous Forest
Wa, Upper West-WAF	10	1075	002°23'018"	10°03'540"	Guinea Savanna
<i>Dactylurina staudingeri</i>					
Kankram-Brong Ahafo-KrD	209	407	001°48'085"	07°40'338"	Transition Zone
Bobiri Forest Reserve, Ashanti-BoD	16	757	001°22'436"	06°39'404"	Deciduous Forest
Koro, Volta-KoD	166	287	000°18'287"	07°24'951"	Deciduous Forest
Abrafo, Central-AbrD	20	131	001°22'393"	05°20'159"	Deciduous Forest
<i>Hypotrigona gribodol</i>					
Kankram-Brong Ahafo-KrH	74	407	001°48'085"	07°40'338"	Transition Zone
Assin Foso, Central-AfH	46	148	001°22'188"	05°20'511"	Deciduous Forest
Assin Akrofuom, Central-AAH	52	140	001°22'227"	05°20'196"	Deciduous Forest
Bobiri Forest Reserve, Ashanti-BoH	160	757	001°22'436"	06°39'404"	Deciduous Forest
Mfonom, Central-MfH	38	192	001°24'216"	05°22'092"	Deciduous Forest
Koro, Volta-KoH	173	287	000°28'287"	07°56'133"	Deciduous Forest

Transition Zone (Derived savanna)

This zone covers about 8300 km² and occurs as a normal strip of about 48 km wide along the north and the north easting limits of the deciduous forest. This zone is also characterized by a bimodal rainfall distribution pattern with mean annual totals ranging from 1200-1400 mm. The mean temperature is about 26.5 °C. Morning and mid-day relative humidity values range between 85.5 and 88% and 70% and 74% respectively. Potential evapotranspiration in this zone is about 1350 mm/yr (40-41). Most tree species of the forest zone occur in this area in addition to such species as *Daniella oliveri*, *Borassus aethiopum* and *Terminalia macroptera*. These trees occur in association with tall to medium grass such as *Andropogon* and *Pennisetum* spp.

Morphometric Studies**Slide Preparation**

Samples of approximately ten workers were taken from each of the colonies, except for colonies from the Transition zone, where 30 workers were used per colony. This was to increase the number of bees within this zone; as few colonies were collected due to time and accessibility constraints.

The right forewing of each worker (total 451 wings) was removed, placed between a microscope slides and secured in position with a microscope cover slide that had a drop of transparent nail polish carefully applied to the ends. In some instances, wings were relaxed in 30% alcohol before mounting unto slides. Each wing was photographed with a digital camera attached to a stereomicroscope (4, 35-36, 42). Wing images were captured and an image file was created in JPEG format. One TPS file was created from the image files using tpsUtil software (version 1.49) (43).

Wing Venation Characters

Eight homologous landmarks were plotted at the junctions of the wing venation of the four stingless bee species using tpsDig2 software version 2.16 (44 (Figure 2)). One TPS file grouped all the eight landmarks derived from each of the 450 wings of the four bee species under study.

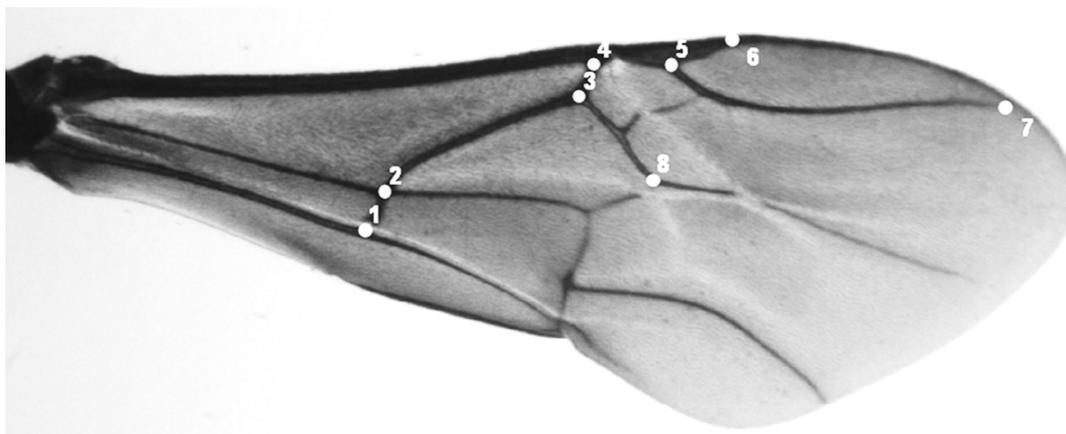


Figure 2. Forewing of stingless bee with eight homologous landmarks

Statistical Analyses

The Cartesian coordinates of the landmarks were Procrustes aligned to evaluate existing shape variations among the different populations using MorphoJ software version 1.03 (45). Within MorphoJ software, further statistical computations including principal component analyses (PCA), canonical variate analyses (CVA), discriminant function analyses (DFA), Procrustes ANOVA and Regression analyses were conducted to discriminate populations within each species against the different ecological zones. In addition, relative warp analyses was conducted to summarize the variation among the specimens (with respect to their partial warp scores) using the tpsRelw software version 1.49 (46). The Mahalanobis square distances between the centroids of CVA were then used to construct a neighbor joining dendrogram with MEGA5 software version 5.05 (47).

RESULTS

Generally, all statistical analyses conducted recorded significant differences within all the species and among the different ecological zones.

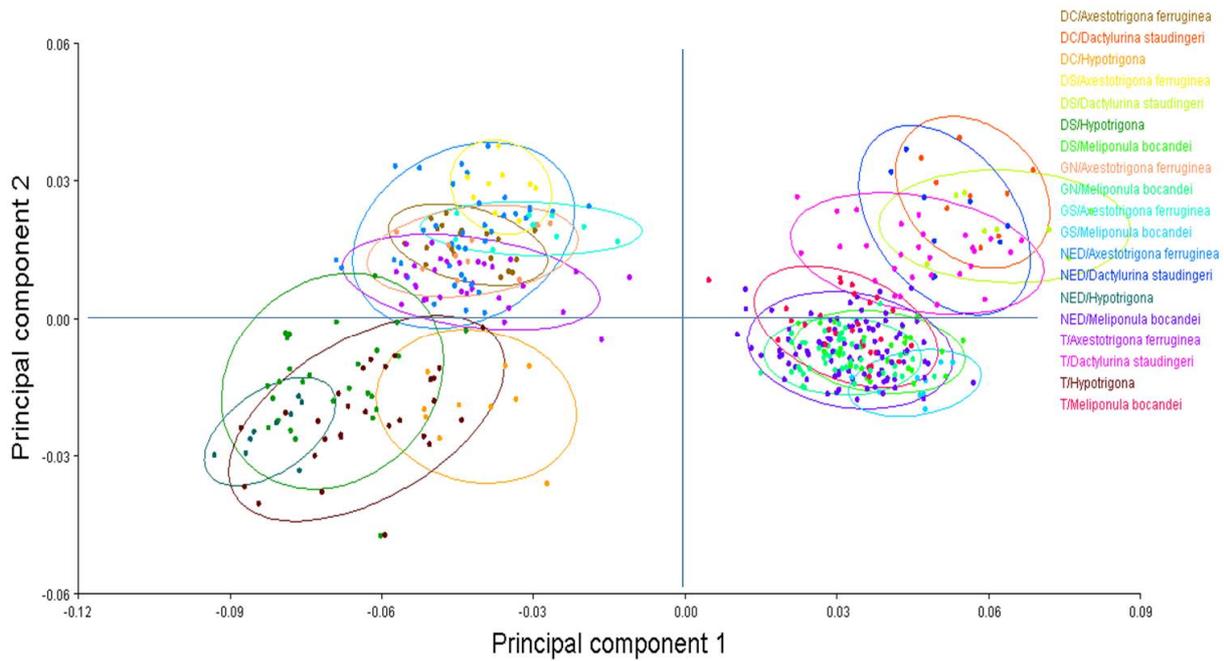


Figure 3. Scatterplot of the Principal Component Analysis showing clustering of the four species within the different ecozones

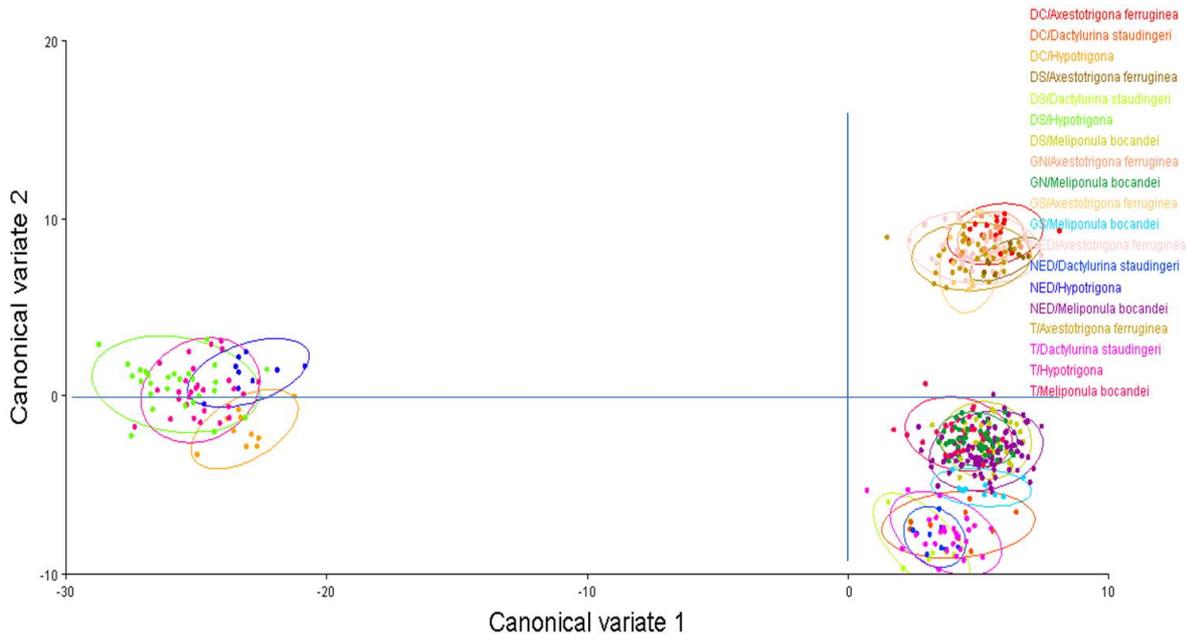


Figure 4. Scatterplot of the Canonical Variate Analysis showing species clusters within ecozones

During the PCA, the first ten factors of eigenvalues were found to be greater than one and these factors accounted for 97.34% of data variability. The three major contributors to this variability are the first three factors (PC 1, PC 2 and PC 3), which explained 62.70% of total data variability, with each factors contributing 32.48%, 16.33% and 13.89 % respectively. Graphical representation of PCA scores shows a number of subpopulations within species and among the ecozones (Figure 3). Species distributions seem to be pulling along latitudinal gradient under the influence of a number of environmental variables.

The Mahalanobis square distances between the centroids of the groups were significantly different among the species and within the different ecozones (Figure 4) with graphical representations showing clear proximities of the various species with each other. *Hypotrigona gribodoi* populations seem to be the farthest from the other three species. Among the other three species, populations of *Meliponula* were closer to the populations of *Dactylurina* than to those of the *Axestotrigona* populations (Figure 5).

The outcome of the dendrogram generated with Mahalanobis square distances between the centroids of CVA revealed probable ecotypes within the various species and the distances between the ecotypes. More than five probable subpopulations are identified within both *Axestotrigona Meliponula* populations. However, four probable subpopulations are identified within *Hypotrigona* populations while two subpopulations are observed within *D. staudingeri* populations (Figure 5).

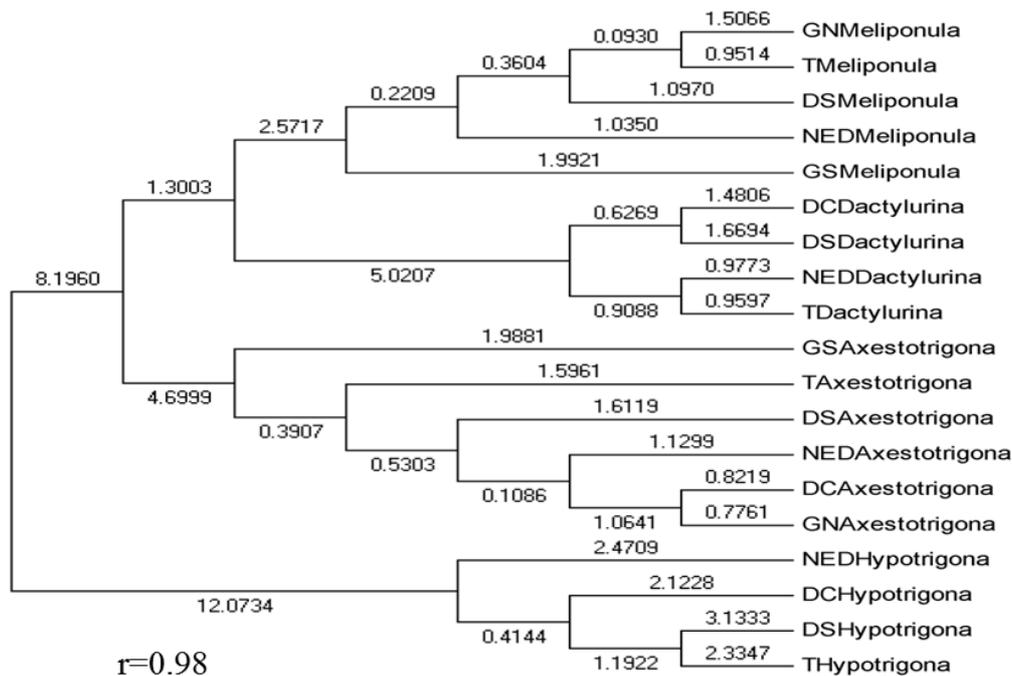


Figure 5. Neighbour-joining dendrogram generated from the Mahalanobis square distances between the Centroids of CVA indicating the probable distribution of species within ecozones

The Discriminant Function Analyses (DFA) also showed highly significant differences within the populations of the different ecological zones with P-values of <0.0001 (Table 2). Graphical representations of the discriminant analyses of each species show a number of subpopulations (ecotypes) more distant from other members of the same species but from different ecozones.

In general, 97.01% of all samples were correctly classified in the respective ecozones during the DFA, with 84.43% of the samples accurately identified in the cross validation test. In *A. ferruginea*, 97.87% DFA scores were able to distinguish the populations within the three ecozones, with 87.25% cross validation. The DF analyses were also able to distinguish between 97.27% of possible “ecological groups” within the *M. bocandei* samples, with 92.45% accuracy in cross validation test. Further to this, the *Hypotrigona* species were 99.71% classified correctly with approximately 91.50% accuracy in cross validation tests, while 94.78% of the populations of *D. staudingeri* populations were correctly classified with 66.54% accuracy. *D. staudingeri* populations recorded the least DFA and cross validation scores.

Table 2. Procrustes ANOVA and Centroid Analysis of the four species

Species	Centroid Sizes		Shape, Procrustes ANOVA									Pillai tr	
	Effect	SS	MS	df	F	P(param)	Effect	SS	MS	df	F		P(param)
<i>Axestotrigona ferruginea</i>	Individual	5214.9979	2607.4989	2	11.43	<0.0001	Individual	0.009356	0.000259	36	11.23	<0.0001	1.21
	Residual	28064.5763	228.1673	123			Residual	0.05123	0.00002	2214			
<i>Meliponula bocandei</i>	Individual	10668.5669	5334.2835	2	9.4	<0.0001	Individual	0.013373	0.000371	36	19.51	<0.0001	1.2
	Residual	119743.1163	567.5029	211			Residual	0.07231	0.00002	3798			
<i>Dactylurina staudingeri</i>	Individual	3522.9247	3522.9247	1	12.54	0.0008	Individual	0.00558	0.00031	18	12.75	<0.0001	0.74
	Residual	17694.8524	280.8707	63			Residual	0.02758	0.00002	1134			
<i>Hypotrigona gribodoi</i>	Individual	8548.4046	8545.4046	1	3.94	0.0504	Individual	0.005839	0.000487	12	9.53	<0.0001	0.74
	Residual	177804.4389	2168.34682	82			Residual	0.050268	0.000051	984			

DISCUSSION

According to Gaston (48), several mechanisms are said to be determinant of biological diversity under the influence of environmental variables correlated with latitude. Within the Order Hymenoptera, environmental variation clearly is the most important determinant of phenotypic variation (49-50). In recent years, human interventions are also playing key roles in the distribution and /or maintenance of species (51-52).

In this present study, geometric morphometrics is able to capture variations among 40 populations of four species of Africa stingless bees (*Axestotrigona ferruginea*, *Meliponula bocandei*, *Dactylurina staudingeri* and *Hypotrigona gribodoi*) and successfully discriminated populations against three ecological zones in Ghana. Each ecozone appears to consist of clusters of subpopulations that are far distant from other ecozones and does reveal possible ecotypes within the stingless bee populations.

Species within ecozones seem to polarize towards specific geographic location (latitudes and longitudes) with similar environmental parameters such as rainfall/precipitation, potential evapotranspiration and vegetation cover. Clustering of subpopulations observed within the graphical presentations of all the analyses (CVA, DFA, PCA) conducted in this study strongly align to this hypothesis. Geometric wing morphometric technique therefore seems to be an effective tool for discriminating African stingless bees just as it has been employed with other bees from other parts of the world. e.g. Neotropical stingless bees (Mendes, et al., 2007; Franco *et al*, 2009, 2011), *Apis mellifera* (34, 36, 39, 53), *Bombus* (54), *Liotrigona* (55), *Euglossa* (37), among others.

In *Axestotrigona* and *Meliponula* spp where populations were analyzed from all the three ecozones, populations from south of Guinea savanna (Kyabobo (Ky)) gravitated towards those in the North East of the Deciduous Forest in all the analyses conducted. One of the most plausible reasons was that the sampling location of the Guinea south contains some patches of remnant forest with similar climatic and vegetation conditions as those in the north east of the deciduous forest. Populations within the Guinea south were also within a protected habitat (Kyabobo National Park) and therefore had minimal exposure to the high levels of habitat destruction that is ongoing in localities harboring populations in Guinean north. Thus with time, these populations probably subdivided with variations in climatic conditions, as has been observed in most species whose populations are faced with either climatic harshness or climatic stability (56-60).

In the genus *Hypotrigona*, this phenomenon is more prominent and one observes subpopulations in the deciduous south (DS) forming a cluster at one end and the deciduous central also forming another cluster at the other extreme of the species grouping. The populations from the North-east of the deciduous forest greatly overlap the populations within the transition zone. In the FAO (2000/1) report, these area do not only share similar latitudinal positions but also similar mean potential evapotranspiration per year. For species of small body size, these environmental conditions seem important in grouping subpopulations. Populations show great diversity within the sampled localities as observed in all the analyses (CVA, PCA, DFA and dendrogram of the Mahalanobis square distances between the centroids). Three probable ecotypes seem to occur within the species among the two ecozones.

The diversity of substrata available for nest building also greatly influences species diversity (61). Habitat fragmentation and degradations can also impact negatively on bee communities (62-65). This possibly accounts for the low variability observed in populations of genus *Dactylurina* among the ecozones as has been the case of *Melipona scutellaris* and *M. beecheii* in Yucatan Peninsula (66-67). The species require building expose nest at mostly high canopy trees in areas with high environmental degradation. A phenomenon that is frequent in the studied areas. Thus, species seems to be subdivided into only two subpopulations; one cluster comprising populations from deciduous south with populations from North east of the deciduous forming another cluster. Variability in sampled populations of *Meliponula* and *Axestotrigona* was high probably due to human interventions in spite of the high levels of their habitat destruction by various stakeholders (51, 68). These two species are the most cultured species in Ghana currently and nest transportation from one area to another by beekeepers is a common practice, possibly increasing inter and intra population interactions. In genus *Hypotrigona*, populations are able to survive in any crevice/whole available, thus are able to colonize different habitat with different environmental conditions.

With a large number of populations of the four species in this research, the Mahalanobis square distances of CVA was very clear in showing the proximity of the groups to each other, which seems to corroborate with the molecular data from Rasmussen and Cameron, (69). Research outcome seems to confirm the hypothesis that stingless bee genus *Dactylurina* is the closest sister group to the genus *Meliponula*. This classification also places genus *Axestotrigona* far from the genus *Meliponula* and this is supported by highly significant p values (<0.0001) in Procrustes ANOVA.

The methodology used in this study seems to be an effective tool in capturing small variations within species and among populations of varied ecological areas. However, additional data from molecular studies of the species as well as increasing the sample size and number of ecozones could possibly improve our understanding of the various interplays within a species at different ecozone.

Of great interest to this research is the essential contribution it will make to conservation strategies of these species. The research results presented here clearly shows that the four African stingless bees comprises at least 16 ecotypes, if not subspecies, therefore treating each species as individual taxon is not appropriate for their development as commercial honey producers and pollinators. The International Stingless Bee Centre in Ghana, through various interventions has trained several scientists and non-scientists in keeping these species for hive products and conservation purposes. Similar efforts are ongoing in many countries in East Africa. Results of this study show it is possible to successfully achieve high yielding outcomes from keeping these species with regards to the ecotypes of the species. We therefore recommend that all stakeholders should consider the results of this study when exploring movement of colonies from one ecological zone to another.

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