DNA BARCODING AND MORPHOMETRICS OF STINGLESS BEES (APIDAE: MELIPONINAE) IN THREE SELECTED FORESTS IN KENYA

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DECLARATION AND RECOMMENDATION

I hereby, declare that this thesis is my original work and has not been presented to any other university or institution for the award of degree.

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Recommendation

This research thesis has been submitted with our approval.

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DEDICATION

This work is dedicated to my parents Mr and Mrs B. N. Choroma and my loving husband Joseph Mucugu for being very supportive throughout my studies.

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ABSTRACT

Stingless honeybee keeping is an income generating enterprise with an indirect potential of achieving the goal of forest and biodiversity conservation in Kenya. However, little information is available on species diversity and spatial distribution of stingless bees in Kenya. This study describes variations of stingless bee samples collected from Arabuko Sokoke, Mwingi and Kakamega forests, ranging from low, middle to high altitude, respectively using morphometrics and DNA barcoding. Fourteen morphometric characters were measured to determine the extent of morphological variation of stingless bees in Kenya. In addition, mitochondrial DNA sequences data were generated using Cytochrome c Oxidase I (COI) in accordance with standards applied by the Barcode of Life Data systems (BOLD). The morphometric variables differentiated three populations of *Hypotrigona gribodoi* and *Meliponula bocandei* from three geographically distinct regions in Kenya. *Hypotrigona gribodoi* samples from three localities were separated using morphometric data by applying PCA (Principal Component Analysis) and CVA (Canonical Variate Analysis) into two population groups. Kakamega population was distinct whereas Mwingi and Coast separated partially. Three populations of *M. bocandei* were separated into two groups on the PCA but separated further into three distinct groups on CVA. Sequences from the same species clustered together when genetic distance- based cluster analysis was applied. Intraspecific divergence was less than 2% compared to high interspecific divergence of greater than 8.6%. The distance between groups analysis shows that *H. gribodoi* is closely related to *D. schimidti* with a distance of 0.086 compared to *M. bocandei* with 0.119. This study forms a basis for further studies in production of queens for commercial production of honey. The production of honey acts as a source of income and involves farmers in conservation of forests.

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Stingless bees (Hymenoptera: Apidae: Meliponinae) are highly social and eusocial insects consisting of over 400 species (Roubik, 1989). The bees live in colonies formed through a process of swarming. Rearing of stingless bees is known as meliponiculture, comparable to apiculture which refers to the rearing of honeybees (Eardley, 2004). Stingless bees are among the oldest known bees and are found in tropical and sub-tropical regions of the world. They have populated the earth for over 65 million years longer than the stinging bees, *Apis mellifera* (Carmago and Pedro, 1992; Michener, 2000). The oldest fossil was found in New Jersey USA, in Cretaceous amber of 96-74 mya and this was a worker of stingless bee *Trigona prisca*, resembling the modern day *Trigona* bees (Velthuis, 1997). The genus *Trigona* is believed to be the oldest group of stingless bees known and the most common species.

Six genera of stingless bees, comprising 19 species, are known to occur in Africa. Michener, (2000) argues that African tropics could be richer in more bee species but lack appropriate data to prove it. In five species (*Dactylurina cockerell, Meliponula cockerell, Plebeina moure, Hypotrigona cockerel and Liotrigona moure*), workers collect pollen and nectar from flowers, and in one species (*Cleptotrigona moure*), workers rob pollen and nectar from the nests of other stingless bees (Eardley, 2004). The genus *Trigona* belongs to sub family Meliponinae that consists of the genera *Austrolebia, Lastrimelitta, Melipona* and *Trigona*. Of these, *Austrolebia*, *Melipona* and *Trigona* species are associated with honey production. In Kenya, *Trigona* species are widely distributed especially in warm naturally forested areas such as Kakamega, Mwingi and Arabuko-Sokoke forests. Twelve species have been identified in different regions in Kenya. In Kakamega the species include, *Meliponula bocandei, M. (Trigona) ferruginea, M. lendliana, Pleibeina hildebrandti, Hypotrigona araujo and M. (Meliplebeina) becarii*; in Mwingi, *Hypotrigona gribodoi, M. (Trigona) ferruginea and H. araujo* and in Arabuko sokoke, *Dactylurina schmidti, H. gribodoi, M. (Trigona) ferruginea, H. araujo* (Eardley, 2004; Gikungu, 2006).

The economic value of pollination by bees exceeds that of honey production Nabhan (1996), yet this data is currently missing in Kenya (Gikungu, 2006). There is an urgent need to document and conserve the abundance and species diversity of the bees in their natural habitats before they are lost as a result of the prevailing habitat degradation. Stingless bees are among the most poorly studied insect groups in many ecosystems of East Africa. The poor knowledge of stingless bee fauna in Africa can probably be associated with several factors, such as lack of bee specialists in the past and few bee collections. The few available bee collections in the local institutions, such as Museums, are therefore incidental collections by either naturalists or ecologists who sampled them occasionally as they studied the targeted insect groups (Gikungu, 2006). Unfortunately, no detailed publications are available on the species collected from any of the habitats in Kenya. Some species documented from Kakamega forest may have disappeared with time. For instance, according to the available list of bees from National Museums of Kenya, *Dactylurina cummingsii* (s*chmidti*) has been found missing (Gikungu, 2006). Roubik (2001) suggests that to detect a decline in pollinators, a minimum of three years is required. According to a recent stingless bee revision in Africa, there are only two species of the genus *Dactylurina*, that is, *Dactylurina schmidti* and *Dactylurina staudingeri*. These are the only stingless bee species in Africa that make vertical exposed combs, and are geographically separated. *Dactylurina staudingeri* (*Gribodoi*) is found among West African species while *Dactylurina schmidti* is found in East Africa (Eardley, 2004).

Stingless bees are essential for pollination of wild plants and agricultural crops (Klein *et al*., 2003; Kremen *et al*., 2004; Gikungu 2006,). They are also increasingly used to improve productivity of commercial crops because they require less handling and maintenance procedures than honey bees which can sting (Souza *et al*., 2008). In Kakamega, Mwingi and Coast regions of Kenya farmers have formed groups to domesticate the *H. gribodoi* and *M. bocandei* species for honey production. However, the farmers have not started commercial pollination services in Kenya. It has been found that stingless bees are better adapted to pollinate plants whose flowers are hidden under foliage, for example, some species like *Scaptotrigona* leave a trail of scent marks on vegetation, grass or even on stones that help to recruit or guide other bees to the flowering plant (Macharia *et al*., 2007). The size of stingless bees ranging from, 2mm to slightly larger than a centimeter allows the bees to forage in very small flowers like the mango tree and passion fruits flowers where honeybees don’t access

due to their large size. With the growing pressure on the environment and the associated loss of honeybees, the need exists for additional pollinator species to be used in agriculture to maintain resilience in food production and improvement of yield, especially in hot, dry areas where honeybees are less abundant. Meliponini could be utilized as surrogates for honeybees. Replacing destructive harvesting of stingless bees with meliponiculture would provide honey for food and medicine, and enhance pollination of both commercial crops and indigenous plants. However, for such an endeavor to be successful it is important to know the identity and geographic range of the bees involved (Eardley, 2004).

Stingless bees are suffering population declines in many areas of the world due to habitat destruction and deforestation (Melendez-Ramirez *et al*., 2002; Kremen *et al*., 2004; Samejima *et al*., 2004; VillaNueva *et al*., 2005; Gikungu 2006; Macharia *et al*., 2007), with some species probably facing extinction. The increasing loss of biodiversity presents an overwhelming challenge and requires the discovery and analysis of biodiversity at a greatly accelerated pace (Smith *et al*., 2005). The conservation planning needs to be based on biodiversity data and this requires taxonomic knowledge (Brooks *et al*., 2004a, b). The total number of stingless bee species is still not well established because of many cryptic species (Michener, 2000; Barni *et al*., 2007). There exists no comprehensive account of stingless bees in Kenya and it is expected that there are undiscovered species with the potential of honey production. This is due to shortage of taxonomists in East Africa and therefore the existing bee collectons are few and therefore not true representative of the stingless bees in Kenya (Gikungu, 2006) In addition, few extensive analyses have been conducted on the population structure of stingless bee species (Waldschimidti *et al*., 2002; Castanheira and Contel, 2005). To our knowledge this study is the first one in Kenya to include molecular work of stingless bees. The data on genetic structure of bee species are useful for conservation purposes because population genetic purity is greatly affected by bee keeping and commercial breeding practices (Souza *et al*., 2008). One way to study population diversity and distribution is morphometrics. Morphometrics is a field that involves studying variation and change in form (shape and size) of organisms to detect evolutionary relationship. Morphometrics has been used in many studies such as Lepidoptera (Di mare and Corseuil, 2004) and more recently in honeybees *Apis mellifera* (Francoy *et al*., 2006).

In addition to morphometric work, stingless bees' DNA barcodes were generated for the three species. DNA barcoding is a molecular technique that involves retrieval of a 650bp

region at the 5’ end of mitochondrial gene Cytochrome c oxidase I (COI). The region is specific to species and can be used to identify organism in the same species. For unknown specimen short DNA sequence from the COI gene is obtained and compared with a library of reference barcode sequences of known identity (Hajibabaei *et al*., 2006). The steps involved in DNA barcoding includes: sampling, vouchering, DNA isolation and sequencing. Although not always in total agreement, both morphological and molecular genetic tools have proven to be powerful methods with which to establish the genetic structure in other bee species such as *Apis mellifera* (Ruttner, 1988; Estoup *et al*., 1996; Franck *et al*., 2001) and Bombus species (Estoup *et al*., 1996; Widmer *et al*., 1998, Widmer and Schimid- Hempel, 1999).

1.2 Statement of the Problem

Classification of stingless bees has been attempted by many workers with different view points; the most recent classification comprises over 400 species worldwide, 19 species in Africa and only 12 species in Kenya. The species have been little studied, resulting into classification that is still largely unresolved. Data on bee diversity, population dynamics and taxonomy are currently lacking in East Africa. The task of identifying and describing new species is achieved through computerized morphological characterization. In addition, molecular studies such as DNA barcoding can significantly facilitate the process to avoid ambiguous results. There is need for further studies on known *Trigona* species and discovery of new species that are not yet described in Kenya.

1.3 Objectives of the Study

1.3.1 Overall Objective

To describe the biodiversity of stingless bees using morphological and molecular tools that will to contribute to conservation of forests in Kenya.

1.3.2 Specific Objectives

1. To determine variations among three populations of *Hypotrigona gribodoi and Meliponula bocandei* species from Kakamega, Mwingi and Arabuko sokoke forests in Kenya using morphometrics.

2. To develop and apply DNA barcoding using Cytochrome c Oxidase I for identifying *Hypotrigona gribodoi*, *Meliponula bocandei* and *Dactylurina schimidti* species.

1.4 Research Hypotheses

1. *Trigona* species from the three forests in Kenya can be differentiated using morphometrics.

2. Stingless bees from various forests in Kenya that are geographically isolated can be identified using barcodes.

1.5 Justification

Meliponiculture (stingless honeybee keeping) is relatively new in Africa. Few studies have been done on the identification, diversity and distribution of stingless bees in Kenya. However, stingless bees play a great role in pollination and production of highly valued honey that is used as a nutritious food with therapeutic value. Currently, identification of stingless bees is by use of morphological keys, nest architecture, morphological features and nesting sites. The identification system can be greatly improved by computerized morphological characterization. In addition, there is need to incorporate DNA based tools along with morphological taxonomy to enhance accuracy and efficiency in the identification system. This forms a basis for production of queens for farmers to help in commercial production of honey thus encouraging conservation of forests.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of Stingless Bees

Stingless bees are classified as follows: Phylum, *Arthropoda*; Class, *Insecta*; Order, *Hymenoptera*; Suborder, *Apocrita*; Superfamily, *Apoidea*; Family, *Apidae*; Sub family, *Apinae*; Tribes, *Meliponini and Trigonini* (Michener, 2000). The sub family *Apinae* is composed of 19 tribes of which *Apini* (stinging bees or honey bees) and *Meliponini* (stingless bees) are the only two tribes that contain species displaying a high level of social behaviors. The total number of species within Meliponini is still controversial but estimated to be about 400 (Carmago *and Pedro*, 1992; Crane 1992; Sommeijer and van Buren, 1992; Michener, 2000). Although the classification of stingless bees has been attempted by many workers with different view points (Sakagami, 1982), the most recent classification of stingless bees, the *Meliponini* comprises 23 genera and 18 subgenera (Michener, 2000). The total number of species is still not well established because of many cryptic species (Michener, 2007). The task of identifying and describing new species is ultimately achieved through comprehensive taxonomic work (Gikungu, 2006). The shortage of bee taxonomists in East Africa has enormously affected the study of bees in the region. Over the years there has been only one active bee taxonomist in Africa (API Plan of Action, 2003). A recent report from Eardley (2004) indicates that out of 129 genera of bees in sub-Saharan Africa, only 19% are currently being revised. It is also notable that the existing bee collections are few and small and are not true representatives of bee fauna in the region. Lack of adequate bee surveys have also led to poor knowledge of bees in Kenya. In addition, most of the bee collections in East African museums and institutions are characterized by mis-identified or un-updated taxonomic identities, thus making comparative studies very difficult. During the current study, it was difficult to compare the past records of bees from Kakamega forest due to the above-mentioned challenges (Gikungu, 2006).

The subfamily Meliponinae has been divided into the tribes Meliponini and Trigonini due to the discovery of a series of valid taxonomic characters relating to the external morphology but reinforced by characters concerned with general biology, nest architecture, the dorsal vessel, the ventral cord and the alimentary canal. The Meliponini are species found in the

Neotropical areas (tropical America) while the Trigonini appear in the tropical area including Africa, Asia and Australia (Michener, 2000).

2.2 Life Cycle of Stingless Bees

Stingless bees undergo a complete growth cycle: Egg; Larva; Pupa and adult (Mackean, 2007). The queen lays eggs in the brood area where the temperatures are about 32 °C. The eggs hatch into larvae after two days. For the first three days after hatching, all the larvae are fed on a protein-rich, milky secretion, called royal jelly, which comes from the salivary glands of workers of a certain age. The grubs in queen cells continue to be fed on royal jelly for the rest of their lives, but those in drone or worker cells are "weaned" onto a mixture of dilute nectar and pollen (Mackean, 2007). If a one-to-three-day old larva is transferred from a worker to a queen cell, it will receive the diet of royal jelly and develop into a queen. Thus, though there is no difference between the eggs and young larvae in queen and worker cells, their different treatment by the workers results in their becoming quite distinct types of bee. There forms a cocoon on the larvae, which are the pupae. The pupae are delicate and therefore need to develop into adults in order to survive. The adult will finally emerge and fly off to feed and mate. Queens and workers develop from fertilized eggs, which are fed differently as larvae. However, drones develop from unfertilized eggs in wide cells (Mackean, 2007).

2.3 Geographical Distribution of Stingless Bees

Based on paleontological and biogeographic data, stingless bees are considered to have their center of origin in Africa and have dispersed to other tropical and subtropical parts of the world (Velthuis, 1997). This hypothesis is also supported by the fact that their primitive species with a well-developed sting system live in Africa (Willie, 1983). The main distribution of stingless bees was described by Kerr (1969). In contrast to the *Apini* (stinging bees), which has an endemic geographic distribution restricted to the Old world, the *Meliponinae* has a wide distribution and is found throughout the tropical and subtropical regions of the world. Most stingless bees are in tropical South and Central America - 260 species in Brazil and 20-40 each in the tropics of Africa, Asia and Australia (Crane, 1992). The Australia genus *Austroplebela* appears related to the African forms (Michener, 1990). The genus *Melipona* has species are found only in tropical America (Roubik, 1989) and have the largest body size, sometimes as big as that of honeybee, *Apis mellifera* (Linnaeus).

Trigona is an extensive genus of which comprise long-winged bees found in tropical and sub tropical parts of the world with some as large as *Melipona*, though the smallest is approximately 2mm (Michener, 2000). The distribution of the various species depends on availability of nesting cavities.

2.4 Economic Importance of Meliponiculture

Stingless bees, *Meliponini* are economically important insects that produce honey and facilitate pollination. They are gentle and easily manageable. They are the major biomass components of nectar and pollen-foraging insects commonly found in many tropical areas (Johnson and Hobbell, 1986). Some Melipona species are essential to pollination of Brazillian Atlantic forest (Kerr *et al*., 1996) and open savannah flora and agricultural crops (Heard, 1999; Klein *et al*., 2003; Kremen *et al*., 2004) and are important for the conservation of world flora and fauna (Melendez- Ramirez *et al*., 2002; Corlett, 2004; VillaNueva *et al*., 2005). The stingless bees are small in size, thus visit small, widely spaced plants with flowering providing slight amounts of pollen and nectar (Velthius, 1997). The bees' colonies are active every day and therefore have sustained impact among the biota (Roubik, 1989; Hansell, 1993). Unlike honeybees, 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 than honey from other bees (Vit, 2001; Garedew *et al*., 2004). The wax is also used for several purposes such as molding, jewellery and binding metals.

The commercial production of honey from stingless bees has been practiced in several areas of Australia and Central America. However there has been no commercial production of this honey recorded in Africa. Currently, there are no figures on the gains of practicing meliponiculture in Africa. Although trials are currently ongoing in Ghana and South Africa, the potential of the newly discovered stingless bees needs to be validated (Gikungu, 2006).There is also need for selective breeding in Meliponiculture for maximum production of quality honey. However little information is available on the diversity and the reproductive behavior of stingless bees.

2.5 Stingless bees in comparison with the honey bees

Stingless bees usually have single queens, store honey, and have morphologically distinct workers and queens, similar to the honeybees (Peters *et al*., 1998). However, stingless bees

differ from *Apis* (stinging bees) in many biologically significant ways. The Meliponinae (stingless bees) do not migrate with change of weather and they produce brood like solitary bees, with an egg placed on top of a food mass in a sealed cell (Roubik *et al.*, 2005). Meliponinae generally do not sting, mate only once and do not use water to cool their nests nor pure wax to build them. They do not freely swarm to form new colonies but must first make new habitation. The males feed on flowers and gravid queens do not fly (Roubik *et al.*, 2005). They also differ in their social organization in that stingless bees queens are produced in large numbers and if a vigorous queen is already present in the nest it kills the new queen (Peters *et al*., 1998).

Stingless bees usually live in nests, in trees cavities found in the warm tropical forests. The nests are immobile fixtures and potentially long-lived. The nests site and architecture are highly visible aspects of their behavior and are therefore applied in stingless bees' identification (Michener, 1974). Species are often recognizable from nest entrances and often much obvious variety exists (Roubik, 2005). Inside the nest, there are separate pots used for storage of nectar or ripened honey (Velthius, 1997). Meliponinae are easily distinguished morphologically from other tribes of the family Apinae by the reduced wing venation, presence of a penicillum (a brush of long setae on the outer apical surface of the hind tibia) and a vestigial sting (Willie, 1979, 1983; Michener, 1990, 2000). A major character that separates Meliponinae from other corbulate tribe is the absence of auricle on the hind basitarsus (Costa *et al*., 2002). The species included in the tribe Trigonini show considerable variation in size, nesting sites and nest architectures (Michener, 1974; Sakagami, 1982).

The female castes, queen and workers differ strongly in morphology (Michener, 1974; Willie, 1979). In contrast to the situation in honey bees (Winston, 1987) and bumblebees (Widmer and Schimid-Hempel, 1999), stingless bees colonies and their female reproductive do not migrate and show reduced dispersal (a few hundred meters at the most) during swarming (Engels and Imperatriz-Fonseca, 1990). Their particular mode of reproduction involves gradual movement of construction and food materials between mother and daughter colonies and prevents long distance dispersal of their maternally inherited mitochondrial genes (Quezada-Euan *et al*., 2007).

2.6 Identification of Stingless Bees

Currently stingless bee identification involves morphological features, nest sites and nest architecture (Eardley, 2004). The stingless bees live in tree cavities, ground and in open, depending on species. *Hypotrigona* species prefer nesting on muddy house walls although some are found on trees. *Meliponula bocandei* prefers trees in the forest, while *M. Lendliana* nests on trees and house walls (Gikungu *et al.,* 2006; Macharia *et al*., 2007). Study done in Kakamega forests in Kenya shows that stingless bees are more abundant in *Tithonia diversifolia* and *Caesalphania decapetela* plants (Gikungu 2006). Compared to their sister group, the honey bees (Apini) that are native to much of the tropical and temperate Old World (Ruttner, 1988), the 400 plus species of pan tropical Meliponini have been little studied, the result being that the classification of the group is still largely unresolved (Carmargo *et al*., 1988; Michener, 2000).

The utility of DNA sequences data to resolve Melipona phylogenetic relationships is still to be evaluated but sequence data from the mitochondria (16S Ribonucleic Acid (RNA) gene have been used previously at higher-level phylogenetic studies within the order Hymenoptera (Cameron, 1991; Derr *et al*., 1992; Cameron, 1993; Koulianos *et al*., 1999; Cameroon and Mardulyn, 2001; Downton and Austin, 2001). The two oriental representatives of *Trigona* senso *Heterotrigona* and *Lepidotrigona* (Michener, 1990) came out as sister groups in the parsinary analysis using mitochondrial DNA markers (Costa *et al*., 2002). Morphological evidence should be interpreted with caution, for example, *Dactylurina* was for a long time considered the only African representative of *Trigona* species because of its elongated body shape, shiny integument, narrow keirotrochiate ridge and plumose setae along posterior margin of the hind tibia (Moure, 1961), however, morphology of the worker sting rudiments and male genitalia lead Michener (1990) to include *Dactylurina* among the other African taxa as the sister group of *Plebeina* (Costa *et al*., 2002).

The huge trees that normally provide nesting sites for bees have, unfortunately, been the target of loggers over the years. During this study, the few colonies of bees such as *Meliponula bocandei* and honeybees recorded are only found in mature forests with huge old trees. However, some cluster builder stingless bees such as *Hypotrigona gribodoi* are found mainly nesting in muddy house walls. Unfortunately, conservation of refugia sites for bees continues to pose a serious challenge. Such sites include forest pathways and roadsides,

which are often cleared without much consideration as to their role in pollinator conservation (Gikungu, 2006).

2.7 Morphological Studies of Bees

The wings venation of stingless bees is greatly reduced, marginal cell is open apically and the distal parts of its veins much narrower than their basal parts near the stigma. The stigma is large but the pre stigma is almost absent. First and second sub marginal cells are often unrecognizable and the third cell is not defined (Michener, 2007). Thus, for morphometric studies, veins of the radial cell and cubital cell were selected for measurements. These cells were present in all three species.

The first attempts to classify bees' subspecies were based mainly on colour and size (Ruttner *et al*., 1978; Francoy, *et al*., 2006). However, these discriminations remained imprecise because the ranges of the features that were measured often overlapped (Ruttner, 1988). DuPraw (1964, 1965a, b) was the first to apply discriminant analysis to a set of quantative characters of wing venation and he correctly classified many subspecies. Studies done by Raina and Kimbu, (2005) on *A. mellifera* in Kenya using ANOVA for the body sizes, abdominal bandings and size indicated that honeybees are different in size from lowlands Mombasa in coastal regions to highlands in Kinangop. Thorough analysis of other members of the Family Apidae (*Apis mellifera, Apis cerana, Apis florae* and *Bombus* species) has revealed patterns of subtle differentiation amongst population (Estoup *et al*., 1996; Widmer *et al*., 1998; Widmer and Schimid- Hempel, 1999; Hepburn *et al*., 2001, 2005; Radloff *et al*., 2005).

Previous studies show that there is strong influence in the morphology of *Apis Mellifera* (Eischen *et al*., 1982; Milne and Friers, 1984; Milne *et al*., 1986). Worker bee size may reflect adaptation to local environmental conditions (Ruttner, 1988). Hepburn *et al*., (2001) and Hepburn and Radloff, (1998) have established empirically that the greater the geographical distances between honey bees populations, the greater the likelihood that such groups will show substantial separation in multivariate analysis of size, though whether its due to selection, genetic drift or both is still unclear. Other studied character, the antennae and the wings (Mattu and Verma, 1983), verified that the total length of the antenna in *Apis cerana indica*, increases in agreement with the increase of altitude (Oliveira-JR *et al*., 2000). There is positive correlation on the length of the wing and the altitude (Meixner *et al*., 1989). The phenotypic correlation can supply information concerning the morphological evolution

of the organism in study (Milnie, 1981). Field experiments show that morphometrics can reveal a high degree of polymorphism among specimen (Dujardin *et al*., 1997). The most recent comprehensive classification of bees assigns stingless bees to the large long-tongued (L-T) subfamily Apinae, which includes 19 tribes, including the commercially important Apini (honey bees), Ctenoplectrini and bombini (bumble bees) (Michener, 2007; Schaefer and Renner, 2008). This placement is supported by the morphological studies using 131 characters of adult bees (Roig-Alsina and Michener, 1993).

2.8 Mitochondrial DNA studies of Stingless Bees

Mitochondrial DNA (mtDNA) has been commonly used as a molecular genetic marker in vertebrates and invertebrates due to its characteristics, such as high-mutation rates, maternal inheritance, absence of recombination, and small molecular size (Brown *et al*., 1979; Moritz *et al.*, 1987; Harrison, 1989; Barni *et al*., 2007). Analysis of mtDNA polymorphism has been used in several animal studies, such as population dynamics, species and subspecies characterization, systematics, and phylogeny (Moritz *et al*., 1987; Patarnello *et al*., 1994; Weinlich *et al*., 2004).

Biodiversity of bees was first assessed using morphometric analyses (Barni *et al*., 2007). Twenty-four subspecies of *Apis mellifera* were recognized and grouped into three or four evolutionary groups (Ruttner *et al*., 1978). This was later confirmed by mtDNA analysis with little modifications to subspecies composition (Estoup *et al*., 1995; Frank *et al*., 2000). The fact that mtDNA is maternally inherited has been applied to investigate the ancestors of individual colonies and to study gene flow. This includes studies such as the spread of African-derived honeybees from Brazil to other parts of America (Shepperd and Smitth, 2000). Mitochondrial DNA has been used in studies of genera Plebia and Melipona resulting in restriction maps for species belonging to this group (Francisco *et al*., 2001; Weinlich *et al.*, 2004).

Melipona sequence data resulting from mitochondria genes used previously at higher-level phylogenetic studies within the order Hymenoptera show that it is possible to resolve *Trigona* species (Cameron and Mardulyn, 2001). The mitochondrial DNA molecular marker, cytochrome B and PCR-RFLP facilitated diagnosis of *Meliponula quadrifasciata* subspecies matrilines and was used to establish their natural distribution and to identify hybrid colonies (Souza *et al*., 2008).

2.9 DNA barcoding

DNA barcoding is a molecular technique that involves retrieval of a standard region of mitochondrial gene, cytochrome c oxidase I (COI) at its 5' end containing ~650 base pairs (Herbert *et al*., 2003). The obtained sequence is specific for a particular species and therefore used as its 'tag' or barcode in identification of organisms in the same species. The amplification of the 650 base pair region utilizes universal primers that are designed to amplify COI gene broad taxa. The two main goals of DNA barcoding are, to assign unknown specimen to species and to enhance discovery of new species and facilitate identification, particularly in cryptic and other organisms with complex morphology (Herbert *et al*., 2003a, b). DNA barcoding is an initiative project by the Consortium for the barcode of life to build a barcode library for all eukaryotic life using Barcode for Life Data System (BOLD). The BOLD is an informatics workbench that aids in the acquisition, storage and publication of DNA barcode records by assembling molecular, morphological and distributional data and bridges the gap between traditional taxonomy and bioinformatics (Ratnasingham and Herbert, 2007).

DNA barcoding employs sequence diversity in short standardised gene regions to add in the large assemblies in the genomic data base that is accessible to the public. A 648 region of the Cytochrome c oxidase I (COI) forms the primary barcode sequence for the animal kingdom (Herbert *et al*., 2003a, b). Although many aspects of DNA barcoding have been critiqued (Sperling, 2003; Moritz and Cicero, 2004; Will and Rubinoff, 2004; Ebach and Holdrege, 2005; Will *et al*., 2005), there has been limited discussion on the use of ~600bp fragment and little has been discussed on the potential effects of using this particular region to delimit closely related species (Roe and Sperling, 2007).

DNA barcoding has been criticized by some authors due to some crucial pitfalls; first, the sampling shortage across taxa can sometimes lead to a barcoding gap thus sampling must cover the major part of the existing diversity (Meyer and Paulay, 2005). Identification difficulties arise when the unknown specimens come from currently under-described part of biodiversity (Rubbinoff *et al*., 2006). Secondly, there is risk due to maternal inheritance of mitochondrial DNA thus leading to over estimation of sample divergence, for example, *H. mermerodes* (Lepidoptera) mtDNA polymorphism is structured according to the host plant on which females feed and the two clades produced by phylogenetic analysis are artifacts of female nutritional choice (Hulcr *et al*., 2007). It is expected that projects that rely on shorter

DNA fragments for delimiting the species to be more vulnerable to heterogenous patterns of nucleotide substitution within COI and it is questionable whether this region and the length is optimally informative. Mutation hot spots or adaptive substitution are known to exist in mtDNA causing heterogeneous evolutionary rates across genes (Stoneking, 2000; Innan and Nodborg, 2002). Current results show that this record will be very effective in the more than 955 species of the animals have been shown to possess distinctive CO1 sequences (Herbert *et al.,* 2003; Ward *et al.,* 2005; Hajibabaei *et al.,* 2006).

In poorly studied taxonomic groups, DNA barcoding can be applied in conventional taxonomic works to quickly sort specimens into genetically diverging groups. For Example, DNA Barcoding has been used understand the biodiversity of the caterpillar fauna in Northwestern Costa Rica (Roubik *et al.,* 2005). This library is now used to speed up the sorting and identification of specimens in concert with the traditional taxonomic workflow. DNA barcoding has been applied in population genetics because mitochondrial DNA markers are haploid and unipolarly inherited; they are frequent target for analyses and have a particularly strong contribution to population level studies. The identification of stingless bees will be greatly improved by use of DNA barcoding along with the morphological taxonomy. Typically, COI sequence divergence between insect species is of the order of 3-10% (Hebert *et al*., 2003a, b; Monaghan *et al*., 2005), though sibling species may show far lower sequence divergence as low as 0.32% (Hebert *et al*., 2004b).

It is evident that ignoring morphology, ecology and behavior in taxonomic work is inappropriate but the addition of molecular barcoding can only assist in correct species identification, or at least raise questions that are relevant to the case at hand. Molecular barcoding has the potential to facilitate the identification of any history stage, sex, tissue or even archived sample (although formalin preserved tissues often render DNA unusable) of the species in query. Further more, the inclusion of a limited number of specimens from geographically separated localities that are likely to be genetically different will contribute to taxonomy and population genetics (Creer, 2005).

2.9.1 Cytochrome c 0xidase I (COI) Region for standard barcoding

The mtDNA is considered suitable molecule for barcodes because it is easy to isolate and sequence, and the molecule evolves at a rate that is often phylogeneticaly informative with respect to lower taxonomic levels (Avise *et al*., 1987; Puorto *et al*., 2001). The mitochondrial

gene, Cytochrome c oxidase I (COI) has been proposed as a candidate locus given its conserved sequences allows for universal primers to be used across multiple divergent taxa and its high degree of phylogenetic signal relative to other mtDNA loci that have been used for interspecific analysis (12S or 16S Ribosomal DNA). This is due to heavy stabilizing selection within species for mitochondrial or nuclear cytochrome protein complexes (Herbert *et al*., 2003a, b; Lorenz *et al*., 2005). The cytochrome oxidase I have emerged as the most promising DNA region for zoological barcoding projects (Schaefer and Renner, 2008). In lower level bee systematics, it is generally used without pseudogenes problems (Danforth *et al*., 2004). In insects, Jordal and Hewitt (2004), excluded putative COI pseudogenes in a beetle study, and Herbert *et al*., (2004b) reported cases of COI heterozygosity in butterflies. Other cases have been reported from sea urchins and copepod (Bucklin *et al*., 2007; Williams and Knowlton, 2001). The pseudogenes could be as a result of mitochondrial DNA transferred to nuclear genome, where the mutation rates are considerably lower than in the mitochondria. This explains why sister species relations are no longer resolved in the pseudogenes phylogram (Schaefer and Renner, 2008). Extremely high levels of mitochondrial- nuclear transfers (NUMTs) have been reported from the honey bee (*Apis mellifera*) (Pamilo *et al*., 2007).

2.9.2 Nuclear Mitochondrial DNAs (NUMTs)

Nuclear mitochondrial DNAs (NUMTs) are nuclear copies of mitochondrial DNA sequences that have been translocated into the nuclear genome (Willams and Knowlton, 2001). In eukaryotes, the number and the size of NUMTs are variable, ranging from none or few in *Anopheles*, *Caenorhabditis* and *Plasmodium*, to more than 500 in humans, rice and *Arabidopsis* (Richly and Leister, 2004). As reported by Ann Bucklin (Oral communication., the 3rd international Conservation Gen. symposium, New York, 2007) using DNA barcoding in investigations on marine zooplankton, and by Lorenz *et al*., (2005) performing primate DNA barcoding, nuclear COI copies can sometimes greatly complicate the straightforward collection of mitochondrial COI sequences. Disturbance due to NUMTs must be seriously considered, in both DNA barcode library construction and further specimen identification (Fre´zal and Leblois, 2008). Owing to their particular codon structure, non-synonymous mutations, premature stop codons and insertion-deletions (Strugnell and Lindgren, 2007), NUMTs can be recognized in the sequence and in the amino acid alignments. In the sequence acquisition stage, NUMTs can be detected by the sequence with inconsistent amino acid

alignment. Only recently integrated NUMTs that are difficult to detect (Thalmann *et al*., 2004), could be ignored. Although it is more difficult, it is nevertheless possible to get the true MtCOI sequence of voucher specimens with the reverse transcription (Collura *et al*., 1996). In the diagnostic stage, there may be cases where NUMT occurrence is unknown, which highlights the care that should be taken in DNA barcode alignments.

2.9.3 Delimitation of species in using DNA barcodes

The most controversial objective of DNA barcode analyses is to classify clusters of individuals and consider them as species, in other words, to do molecular taxonomy on unidentified taxa (Fre´zal and Leblois, 2008). The molecular delimitation is much more complicated than assignment to a pre-identified taxonomic group. Herbert *et al*., 2004b proposed the use of a divergence threshold to delimit species. The underlying idea was that intraspecies divergence is lower than inter-species divergence. The standard divergence threshold value advised was of ten times the mean intraspecific variation (‘10-fold rule’) with the reciprocal monophyly. Despite the efficiency of the threshold approach reported for fishes, (Ward *et al*., 2005), crustaceans (Lefebure *et al*., 2006), North American birds (Herbert *et al*., 2004b), tropical lepidopterans (Hajibabaei *et al*., 2006) and cave-dwelling spiders Paquin and Hedin, 2004), the use of thresholds in species description has been strongly discouraged. Indeed, the divergence- threshold methods lack strong biological support and undoubtedly could not become a universal criterion suited to animal species delineation (Meyer and Paulay, 2005; Hickerson *et al*., 2006; Weimer and Fiedler, 2007).

In their literature studies of mitochondrial DNA on low taxonomic-level animal phylogeny, Funk and Omland, (2003) detected species-level paraphyly or polyphyly in 23% of 2319 assayed species, demonstrating that Neighbor Joining-tree analysis will fail to assign query sequences in a significant proportion of cases (Ross *et al*., 2008). It is therefore important to clearly characterize the proportion of non-monophyletic species and the relationship between intra- and inter-specific variability in various taxa to globally assess the relevance of such threshold approaches. Every taxonomic decision using DNA Barcode data should be validated by other independent lines of evidence (Frezal *et al*., 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sampling

Stingless bee samples (143), were collected from three different regions in Kenya, namely Arabuko Sokoke Forest (ASF) (55) (below 500m), Kakamega Forest (KF) (38) (1000-2500m) and Mwingi (500-1000m) (50). Global positioning system (GPS) coordinates were taken at each site (Table 1). Three stingless bees species *(Dactylurina schimidti*, *Hypotrigona gribodoi and Meliponula bocandei.*) were collected from different locations in the three ecological zones; Kakamega (Isiekuti, Ileho & Vuyuya), Mwingi (Kasanga, Nguni & Ngomeni) and Arabuko-sokoke (Dida, Gede, Msitu & Tanariver) forests in Kenya. All samples were preserved in absolute ethanol until morphometric and molecular analysis were done.

3.2. Morphometric study

3.2.1 Slide preparation and Measurements

Slide preparation procedures followed the general processes used for fruitflies by Billah *et al*., (2005). The stingless bee samples were dissected under microscope to remove the right fore wing and right hind leg. The legs and wings were mounted on 2mm slides in Canada balsam and dried in the oven at 37^{o}C for 3-6 days. Images of mounted specimen were taken using (Leica EZ4D-Leica Microsystems (Swisterland) limited) microscope. Measurements were taken using LAS EZ version 1.4.0 software to an accuracy of 0.001mm. Fourteen Morphometric characters were chosen in accordance with Hartfelder and Engels (1992); Diniz-Filho and Pignata (1994) and Quezada-Euan *et al*., 2007. They were fore wing length (WL); fore wing width (WW); V3-V8 representing veins on the fore wing; tibia length (TL); Tibial Width (TW); Femur Length (FEL); Stigma length (SL); Stigma Width (SW) and Basitarsus Width (BW) (Fig. 1 and 2). Readings were recorded in three replicates for accuracy. All images were taken at magnification of X20. Voucher specimens were deposited in *icipe's* biosystematics collection.

Table 1: Areas of sampling and their respective Geographical Positioning System (GPS).

		Global Positioning System (GPS)		
Area	**Localities**	**Latitude**	**Longitude**	**Elevation (metres)**
Mwingi	Kasanga	S00 48.536	E038 18.978	626.4
	Nguni	S00 48.536	E038 08.575	979.5
	Ngomeni	S00 37.016	E38 22.070	947.1
Kakamega	Bunyore	N00 19.403	E34 05.853	1180
	Isiekuti	N00 19.107	E039 47 097	1177
	Ileho	N00 19.403	E34 05.853	1191
	Vuyuya	N00 19.107	E039 47 097	1174
Coast (Arabuko sokoke forest)	Dida	S03 22 19.6	E039 47 097	142
	Gede	S03 18 23.7	E039 59 55.3	20
	Tanariver	S02 14 11.4	E040 1041.2	13

Sampling in three forests (Mwingi, Kakamega and Arabuko Sokoke). The location, Gobal Postioning System coordinates and altitude (Elevation) were recorded. Kakamega forest is a high altitude area whereas Mwingi and Coast are low altitude areas. Sampling was done in different localities to ensure wide coverage of diversity.

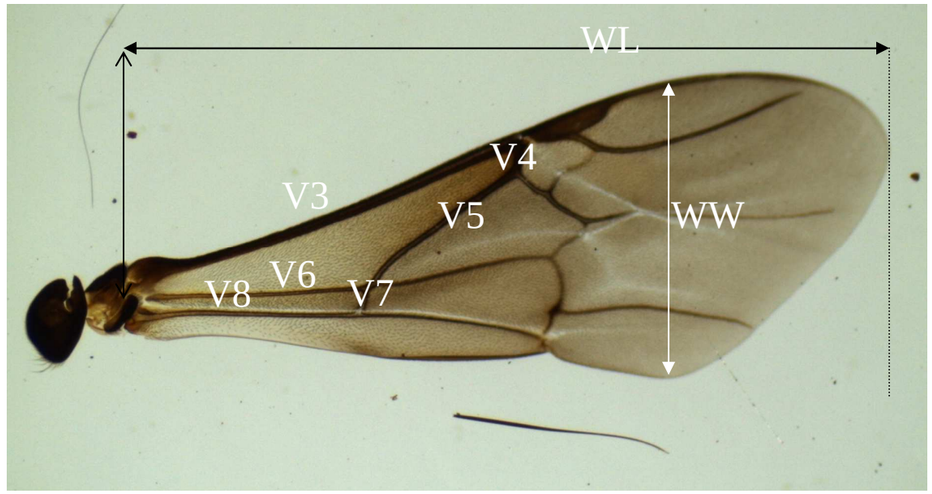

Fig. 1**:** Right fore wing showing veins used in Morphometric studies: WL-Wing Length, WW-Wing Width, V3=Radial vein (R); V4= Rs; V5=basal vein (M); V6= Basal –Cubital vein (M+Cu); V7=Cu- V and V8 =V (anal vein).

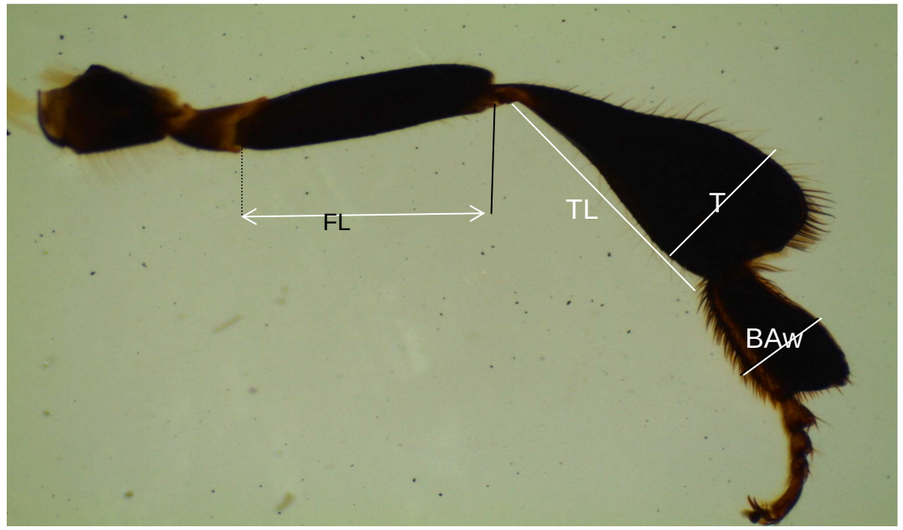

Fig. 2: Right hind leg of stingless bee: FL=Femur Length, TL= Tibial Length, TW=Tibial Width and BAW= Basitarsal width

3.3 DNA Barcoding

3.3.1 DNA Extraction

Genomic DNA was extracted from individual bees using DNeasy Blood and Tissue extraction kit (Qiagen, Gmbh-Hilden, Germany) according to the manufacturer's specifications. The extracted DNA was stored at –20 ^{0}C until required for amplification.

3.3.2 Agarose gel electrophoresis

A 1.8% agarose gel (w/v) was prepared by dissolving 1.8 mg of agarose powder (Sigma – Aldrich Chemie, Gmbh) into 100 ml of 1X TAE buffer (1X working solution buffer is prepared from 10X stock solution (Maniatis *et al*., 1982). The gel solution was stirred, brought to boil in a microwave for 3 minutes to completely dissolve the powder. 5 µl of Ethidium bromide (Ethbr.) per 100 ml of TAE buffer used. Ethbr (Nucleic Acid gels stain) was added to facilitate visualization of DNA under UV light. After cooling the solution to about 60^{0}C it was poured into a casting tray containing sample combs then allowed to solidify at room temperature. After the gel sets, the combs were removed and the gel, on plastic tray was inserted horizontally in the electrophoresis chamber and just covered with 1X TAE buffer. DNA samples were mixed 5:1 with the Blue/Orange 6X loading buffer (6X loading buffer has composition of 15% Ficoll®400, 0.03% Bromophenol blue, 0.03% xylene cyanol FF, 0.4% Orange G, 10Mm Tris-HCl and 50Mm EDTA and loaded into wells. The xylene cyanol FF migrates through agarose gel at the same rate as a double stranded DNA fragment of 4kb, bromophenol at approximately 300bp and orange G at approximately 50bp. A PCR size marker was co-loaded in the first well to determine the size of the fragments. UV Trans-illuminator was used to view the results of gel electrophoresis. A voltage of 80 volts was applied and the samples were run for 1 hour. Images of the DNA bands were produced on a computer.

3.3.3 Polymerase Chain Reaction (PCR) and Sequencing

DNA extracted from the legs of the 92 samples of the 3 species of the stingless bees collected from the three forests (Kakamega, Mwingi and Arabuko Sokoke) was used as template for Polymerase Chain Reaction (PCR). PCR was carried out using universal Forward primer (LCO1490) 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse

primer (HCO2198) 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al*., 1994). The PCR amplification was carried out in 25µl volumes. Each 25µl total PCR reaction contained 5µl, 5X Phusion ^{TM} HF buffer, 0.5µl, 10mM dNTPs, 2 µl DNA template, 0.5 µl, 50mM MgCl2, 0.2 µl, 5U/µl Phusion ^{TM} High-fidelity DNA Taq Polymerase and 1 µl of each forward and reverse primer. PCR standard cycling conditions of 3 min at 94°C, then 35 cycles of 30 s at 94°C, 30 s at the annealing temperature of 47°C and 30 s at 72°C, followed by a final elongation step of 10 min at 72°C were used. The PCR amplification products were analysed by agarose gel electrophoresis. The PCR products were purified using QIAquick PCR purification kit (Qiagen) according to manufacturer's specifications.

3.4 Data Analysis

3.4.1 Morphometrics analysis

Morphometric analysis was performed using statistical analysis system version 9.1 (SAS Institute Inc., 2003) software. For multivariate analysis, Principal Components Analysis (PCA) and Canonical Variate Analysis (CVA) were applied to detect the linear relationships of the species and regions. The principal component analyis was performed on the variance-co-variance matrix for the 10 wing variables (log transformed) to determine the effects of size and shape on the distribution scores along the first two principle component axes (Solkal and Rholf, 1995) and observe their distribution without constraints of prior assignment to particular populations. The data matrix was also subjected to canonical variate analysis to visualize shape differences and evaluate the values for discrimination among populations. Leg measurements were arcsine transformed before being analyzed using General linear model procedure (PROC GLM; SAS Institute Inc., 2001). Significant ($p<0.05$), means was separated using Student-Newman-Kleus (SNK) test.

3.4.2 DNA Barcoding Analysis

Purified PCR products were sequenced in both directions and the resulting sequences assembled and edited using ChromasPro version 1.34 (Technelysium Pty Ltd.). The consensus sequences were aligned in Clustal X version 1.81. The aligned sequences were submitted to the Barcode of Life database (BOLD) (www.barcodinglife.org). The sequences were submitted to BOFAS (Bees of the World - Africa (Stingless bees) database .Accession numbers BOFAS001-08-BOFAS090-08 (http://www.barcodinglife.org/views/projectmenu).

In order to validate the COI sequences obtained in this study were compared to COI sequences from Meliponini for which the whole mitochondrial DNA sequences has been generated and deposited in GenBank. Sequence divergences were calculated using the Kimura 2-Parameter (K2P) distance model (Kimura, 1980), boot strap values were based on 1000 replicates and neighbor-joining (NJ) tree (Saitou and Nei, 1987; Howe *et al*., 2002) was created to provide a graphic representation of among species divergences using MEGA 4.0.2 (Kumar *et al.,* 2008). Pair wise distances were also calculated using Kimura 2-parameter model using MEGA 4.0.2.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 DNA Barcoding

All samples were amplified using universal primers LCO1490F and HCO2198R (Folmer *et al*., 1994) which amplified the 5'-region of Cytochrome c Oxidase I (COI). PCR products generated from 92 samples were visualized on 1.8% agarose gel. The band sizes were between 600-700 base pairs (Fig. 3). All samples met the required DNA barcoding data standard length of at least 500 base pairs (Ratnasingham and Herbert, 2007). The DNA sequences of the three species differed in base pair alignment as illustrated by a selected part of the whole sequence (Fig. 4). In addition to checking clustering for an expected phylogenetic signal, to determine whether a sequence was derived from actual COI and not a numt (nuclear mitochondrial DNA sequences), the sequences were translated to detect the presence of stop codons ('jumping genes'). Sequences which yielded amino acid translation interrupted by stop codons were not included in the analysis as they would not likely be derived from the functional mitochondrial COI gene. In addition, DNA blasting analysis was done to verify whether the fragment were identical to or at least clustered with appropriate whole mitochondrial sequences obtained from GenBank. In cases for which there are no previously reported data to compare with the generated sequences; the extent to which the sequences cluster with members of the same subfamily was tallied. The sequences have been deposited in Bees of the world-Africa (stingless) (BOFAS) project. Accession numbers (BOFAS001-08 -BOFAS090-08) (http://www.barcodinglife.org/views/projectmenu.)

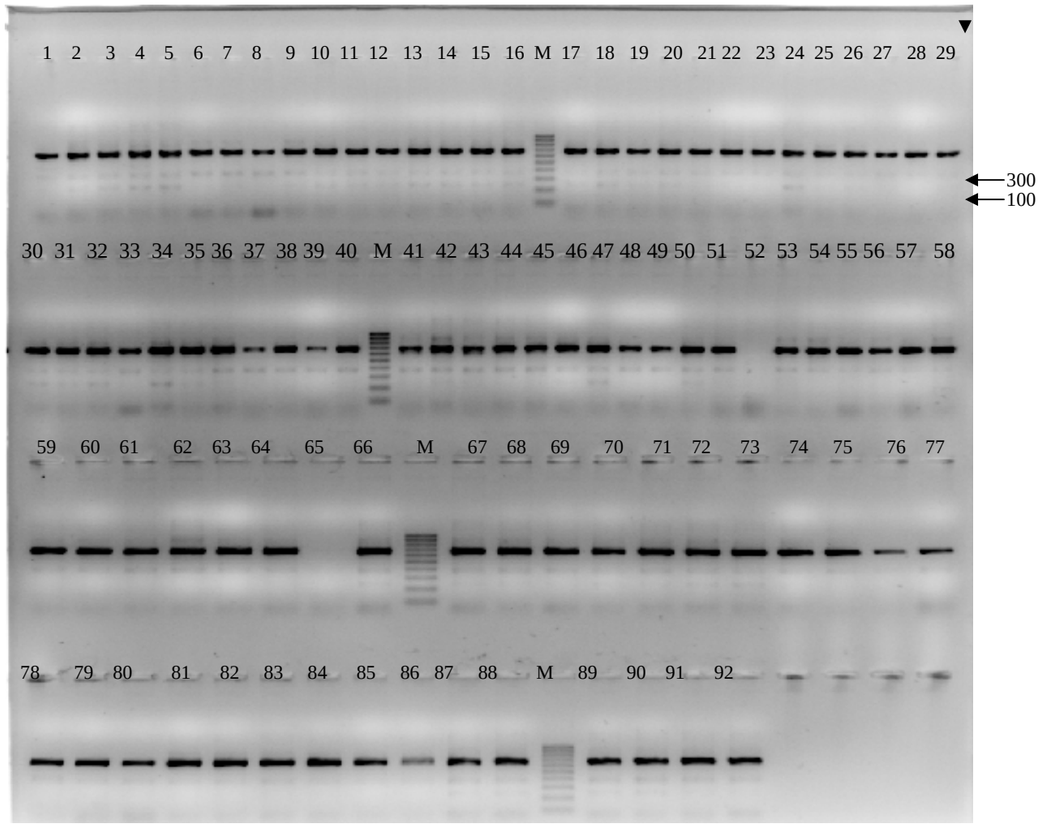

Fig. 3: Amplified Cytochrome Oxidase I sequences from three species of stingless bee sampled from different regions in Kenya: PCR products were visualized in 1.8% Agarose gel. The bees were sampled as follows; Mwingi area *Hypotrigona gribodoi* samples in lane 1-35, (1-10 =Nguni, 11-25=Kasanga; 26-35=Ngomeni); Coastal region *Dactylurina schimidti* samples in lanes 36-40, In Mwingi, *H. gribodoi*=41-50; Kakamega, *Meliponula bocandei* samples in lanes 51-60=Ileho and *H. gribodoi* in lanes 61-65=uvuya.; Coastal region in lanes 66-92, *H. gribodoi* 66-75=Dida, *M. bocandei* 76-80=Dida, *H. gribodoi*, 81-85=Dida and lastly *H. gribodoi*, 86-92=Gede.

M is a 100 basepairs (bp) molecular size marker /ladder

Fig. 4: Nucleotide sequences aligned using ClustalX for the three different stingless bee samples. The bases were selected from 166bp-246bp positions. The encircled bases are areas that the species differ in base alignment. The difference in the DNA sequence alignment differentiates the three species.

Analysis using BOLD managed system resulted into a frequency distribution histogram. At least 95% of the sequenced PCR products from the 92 samples had 680 base pairs nucleotide length with the rest (1-5%) composed of at least 650 base pairs (Fig. 5). Mean base frequencies were G = 9.46%, C = 12.4, A = 32.4% and T = 45.7%. A phylogenetic Neighbor-joining tree was constructed using MEGA 4 (Kumar *et al*., 2008) from representatives of each population group, which were similar in nucleotide sequences. Stingless bees from different localities clustered very closely depending on the region from which they were sampled (Fig 6 & 7).

The neighbor-joining (NJ) tree separated the samples into three groups. Group 1 contained *H. gribodoi* samples, which further separated into three groups, Coast (Arabuko-sokoke), Mwingi and Kakamega. In group 1, *H. gribodoi* samples from Coast (Dida1, Dida2, Dida4 & Gede) and Kakamega (Uvuya), clustered closely. Mwingi samples branching from the same node to form a monophyletic clade, the Kasanga and Ngomeni were clustered closely compared to Nguni samples which seems to separate from the two (Kasanga and Ngomeni). (D=0-0.044 distance). This results depicted that *H. gribodoi* from geographically isolated areas can be discriminated using barcoding. Group 2 contained *M. bocandei* samples as a monophyletic group, Ileho1 and dida3 clustering together but Ieho2 was standing alone. (D=0.03). Lastly, group 3 contained *D. schmidti*, sampled from Coast (Tanariver). *Dactylurina schmidti* forms a branch from the same node with *M. bocandei* samples, that is, they formed a paraphyletic clade (Fig 7).

Analysis of between group average distance calculations using Kimura 2-parameter showed that group 1 (*H. gribodoi*) and group 2 (*M. bocandei*) differed from one another by a distance of 0.111-0.118; group 1 and group 3 (*D. schmidti*) with a distance of 0.084 and group 2 (*M. bocandei)* and group 3 (*D. schmidt*i) a distance of 0.101. *Hypotrigona gribodoi* populations from Mwingi closely clustered to Kakamega samples (D=0.033), than to Mwingi samples (D=0.036). *Dactylurina schmidti* was closer to *H. gribodoi* (D=0.083). Group 2 samples separated further into two groups, that is, Mbileho1 and MbDida (D=0.054) (Table 2). *Dactylurina schimidti* is found in coastal Kenya (Eardley, 2004).

The results were comparable to the BOLD Taxon ID tree constructed using Kimura 2-parameter model (Fig. 6). The NJ tree separated the samples into two main branches, *M. bocandei* in one branch and *D. schmidti* and *H. gribodoi* on the other branch. *Hypotrigona gribodoi* separated further into two smaller groups; group 1 consisting of Mwingi and group

2, Coast and Kakamega samples. *Hypotrigona gribodoi* populations from Mwingi formed a monophyletic clade, however, *H. gribodoi* populations from Arabuko sokoke and Mwingi were paraphyletic. *Dactylurina schmidti* branches from the same node with *H. gribodoi*. Lastly, *M. bocandei* stands in their own branch with Kakamega samples clustering together then the coast samples (Fig 6). Result from bold system management and analysis using pair wise alignment for Nearest Neighbor (NN) showed that the distance between *H. gribodoi-D. schmidti* was 7.53 and *M. bocandei- D. schmidti,* NN distance was 9.06, Standard error (SE)=0.24. The intra specific divergence was 0-3.88, with a mean of 2.14, SE=0.54. Average intra- and interspecific divergencies were 2.14% and 8.04% respectively. The intraspecific variation within species was therefore low compared to interspecifiic divergence between species.

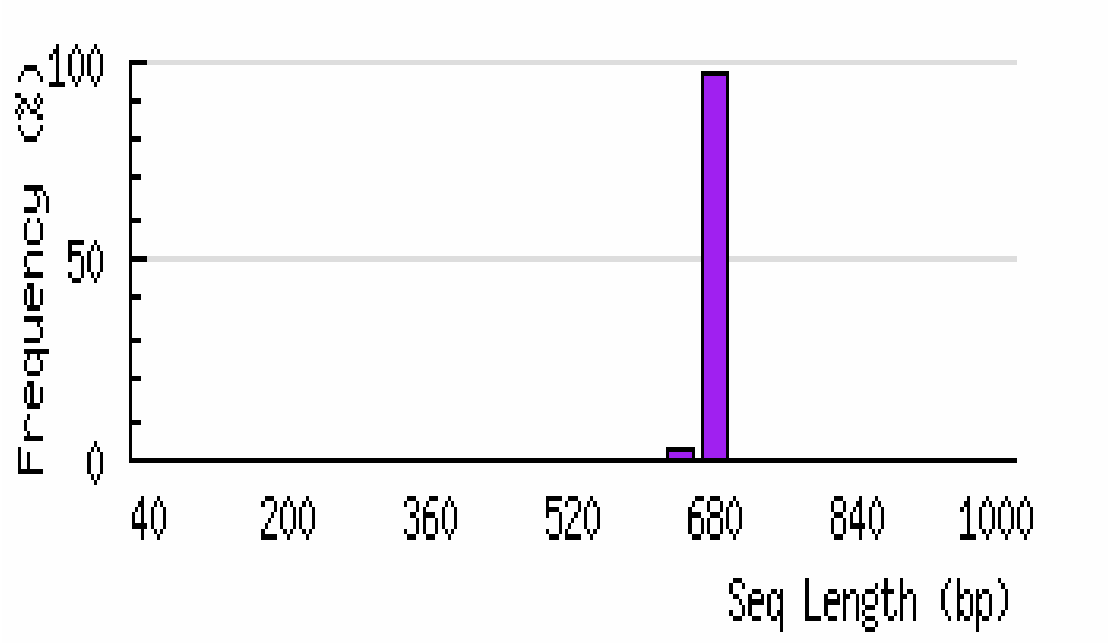

Fig 5: Frequency histograms of sequence length distribution obtained from Barcode of Life managed analysis system: More than 95% of the sequenced PCR products from the 92 samples had at least 650 base pairs with the rest being composed of at least 600 base pairs.

Fig. 6: Neighbor joining (NJ) tree of Cytochrome c oxidase I (COI) sequences. Model=Kimura 2-parameter: The three column represents, species name/ sample number/Country and the region/location, for example, *Meliponula bocandei*=species, S51=sample number 51 and Kenya=Country, Western=Kakamega.

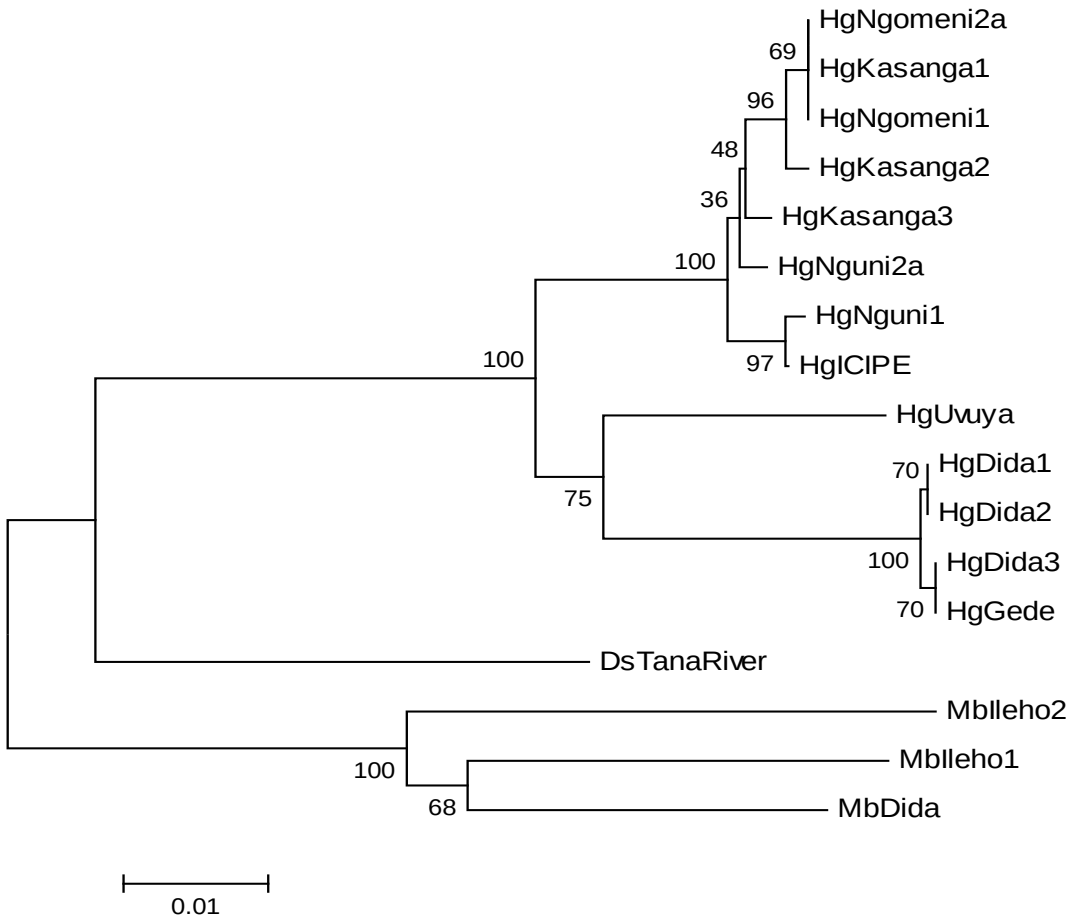

Fig. 7: Bootstrapped Neighbor-joining tree calculated from Kimura 2-p distances and based on 1000 replicates of COI sequences from stingless bees. Scale=K2P distance using MEGA 4: Names codes as follows; species names (Hg=*Hypotrigona gribodoi*, Ds=*Dactylurina schimidti* and Mb=*Meliponula bocandei*). Samples were collected from Mwingi from different localities (Nguni, Ngomeni and Kasanga); from Kakamega (Uvuya and Ileho) and lastly from Coast (Dida, Gede and Tanariver).

Table 2: Pairwise distance calculations computed using Kimura 2 model by MEGA4 .0

Species Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
[1]-Hg Nguni1	-																	
[2]-Hg Ngomeni2b	0	-																
[3]-Hg icipe]	0.002	0.002	-															
[4]-Hg ngomeni1	0.011	0.011	0.009	-														
[5]-Hg ngomeni 2a	0.011	0.011	0.009	0	-													
[6]-Hg Kasanga1]	0.011	0.011	0.009	0	0	-												
[7]-Hg Kasanga2a	0.011	0.011	0.009	0.003	0.003	0.003	-											
[8]-Hg Nguni 2b	0.009	0.009	0.008	0.002	0.002	0.002	0.005	-										
[9]-HgKasanga3	0.011	0.011	0.009	0.006	0.006	0.006	0.006	0.005	-									
[10]-HgNguni2a	0.008	0.008	0.006	0.006	0.006	0.006	0.006	0.005	0.006	-								
[11]-Hg Dida 1	0.047	0.047	0.045	0.045	0.045	0.045	0.045	0.044	0.044	0.044	-							
[12]-Hg Dida 2	0.047	0.047	0.045	0.045	0.045	0.045	0.045	0.044	0.044	0.044	0	-						
[13]-Hg Dida 3	0.047	0.047	0.045	0.045	0.045	0.045	0.045	0.044	0.044	0.044	0.002	0.002	-					
[14]-Hg Gede	0.047	0.047	0.045	0.045	0.045	0.045	0.045	0.044	0.044	0.044	0.002	0.002	0	-				
[15]-Hg Uvuya	0.044	0.044	0.042	0.044	0.044	0.044	0.044	0.042	0.041	0.041	0.042	0.042	0.042	0.042	-			
[16]-Ds Tanariver	0.084	0.084	0.084	0.084	0.084	0.084	0.084	0.082	0.079	0.08	0.092	0.092	0.094	0.094	0.085	-		
[17]-Mb ileho 1	0.111	0.111	0.113	0.118	0.118	0.118	0.118	0.117	0.113	0.115	0.124	0.124	0.126	0.126	0.12	0.101	-	
[18]-Mb Dida	0.113	0.113	0.111	0.113	0.113	0.113	0.113	0.111	0.108	0.11	0.118	0.118	0.12	0.12	0.117	0.099	0.054	-
[19]-Mb ileho 2	0.117	0.117	0.117	0.12	0.12	0.12	0.12	0.118	0.115	0.115	0.129	0.129	0.131	0.131	0.126	0.103	0.07	0.065

Samples were selected from different localities; Mwingi (Nguni, Ngomeni and Kasanga), Coast (Tanariver, Dida and Gede) and Kakamega (Uvuya, Ileho). There are 19 samples selected to represent a group of samples with similar sequences. Names were coded to represent species name then the location of sampling for example, (1-Hg Nguni1 means, *Hypotrigona gribodoi* sampled from Nguni in group 1). Hg =*Hypotrigona gribodoi*, Ds = (*Dactyrulina schimidti*) and Mb= *Meliponula bocandei*. The less the distance the more related the samples.

4.1.2 Morphometric studies

4.1.3 Principle Components and Canonical Variate Analysis

Hypotrigona gribodoi samples were subjected to principle component analysis; from the principal axis plots, the samples were separated into two groups, Kakamega samples appear to be separated from Mwingi-Coast samples (Fig. 8). The first and second Principle Components (PC) contributed 80.85% to the total variation (PC 1=58.27%, PC 2=22.58%). Third, fourth and fifth components contributed; 6.4%, 5%, 4.3%, respectively. Canonical variance separated the three populations into two groups same as principal components. Mwingi-Coast samples could not be separated by either principle component or canonical variate axis. The first and second canonical variates contributed (92.97% and 7.03%), adding up to a total of 100% contribution to the variation (Fig. 9). When *M. bocandei* samples were subjected to principles axis, the three populations separated into two groups Mwingi-Coast and Kakamega (Fig. 10). The first two principle components contributed 61.99% to the total variation (Prin1=39.86%: Prin2= 22.13%). Third, fourth and fifth components contributed (15.83%, 9.25% and 7.63%) respectively to the total variation. On the other hand, when the populations were subjected to canonical variate axis, they separated into three distinct groups (Fig. 11). Canonical variates 1 and 2 were responsible for separation of the populations (CAN1=69.79%, CAN2=30.21%).

The weights of the first principle components for *H. gribodoi* were positive ranging from 0.023-0.99 (Table 3) while for *Meliponula bocandei*, the weights were negative -0.840- 0.477 however the second principle component weights were positive, 0.090-0.573. The major veins contributing to this are stigma V7 and V4 contributing (0.99, 0.069 respectively) and the variables contributing least in the variations are V3 and V6.

Raw, standardized and total canonical structure coefficients of *H. gribodoi* and *M. bocandei* were also computed and are summarized in Table 5 and 6 respectively. *Hypotrigona gribodoi* values for CV1 in total Coefficient Structure (CS) were positive ranging from 0.31-0.9 while CV2 values were negative ranging from -0.66-0.19 (Table 6). For *M bocandei* total CS value were ranging from -0.62-0.78 while in the CV2 the values ranged from -0.4-0.002 (Table 6). The product of pooled within class standard deviation and the canonical vector for each variable is indicated as the standardized canonical coefficient

(Heraty and Wooley, 1993). This represents the amount of change in the original variable by one standard deviation (Neff and Marcus, 1980).

Table 7 and 8 represent Mahalanobis squared distance (D^2) for *H. gribodoi* and *M bocandei* respectively. Mahalanobis squared distance (Mahalanobis A 2 or D^2) is a measure of divergence or distance between groups in terms of multiple characteristics. One can compute it by doing principle components analysis and dividing the eigenvector lengths by their eigen values and summing them. This represents a sum of independent F-tests. Mahalanobis proposed this measure in 1930 in the context of his studies on racial likeness (Mahalanobis, 1930). *H. gribodoi* distance (D^2) was as follows, between Coast and Mwingi populations 2, Kakamega and Coast, 21.3 and Mwingi and Kakamega, 23.15 (Table 7). For *M. bocandei,* Coast and Mwingi, 6.77; Kakamega and Mwingi, 6.85 and lastly between Coast and Kakamega, 13.69 (Table 8).

Mean linear measurements and ratio calculations of leg measurement for *H. gribodoi* populations have been summarized in Table 10. *Hypotrigona gribodoi* from Mwingi had the highest Wing Length (WL) of 2.92mm, then Coast and Kakamega with mean WL of 2.87 and 2.65 respectively. For mean Wing Width (WW), it was in the same order, Mwingi, Coast and Kakamega, with 1.21, 1.2 and 1.13 respectively. The mean Femur Length was longest in Coastal populations (0.8), compared to Mwingi and Kakamega, with 0.78mm and 0.7mm respectively. Mean ratio measurements was significant for V6V3, with an F statistic of 15.16 and d, f of 2, 90. V6V3 ratio was Kakamega, Mwingi and Coast populations, 1.47, 1.41 and 1.37, respectively.

The mean linear measurements for *M. bocandei* were not significantly different, for example, the mean WL for Kakamega, Mwingi and Coast populations were, 5.36, 5.35 and 5.27 and respectively. For FL, Mwingi had the highest mean, then Kakamega and Coast, that is, 1.54mm, 1.53mm and 1.52mm respectively. The mean ratio calculation shows that Coast and Kakamega populations are similar, the WWWL ration seem to be equal in all, that is 2.56 , however V6V3 ration seem to differ in Kakamega and Coast (1.72 and 1.67) respectively, while in Mwingi it was the highest 1.75 (Table 9 and 10).

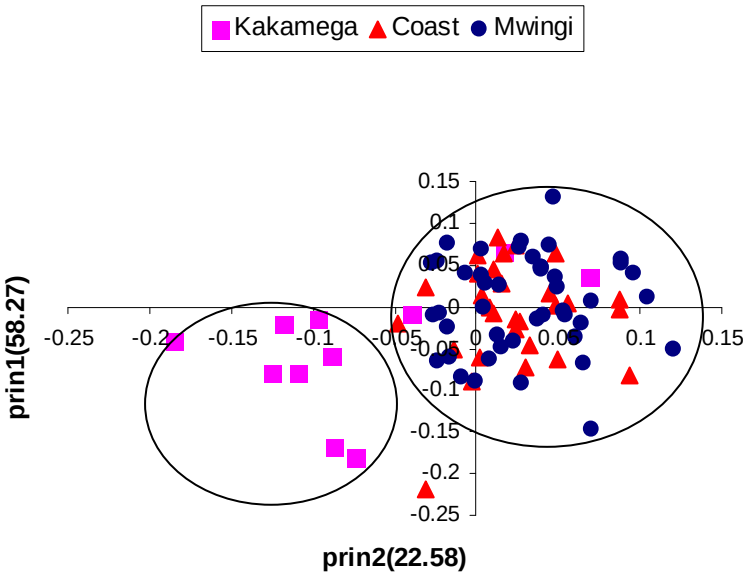

Fig. 8: Projection of wing vein data on first two principle components for *H. gribodoi* species population collected from Kakamega, Mwingi and Coast. Kakamega population separated from Mwingi-Coast populations.

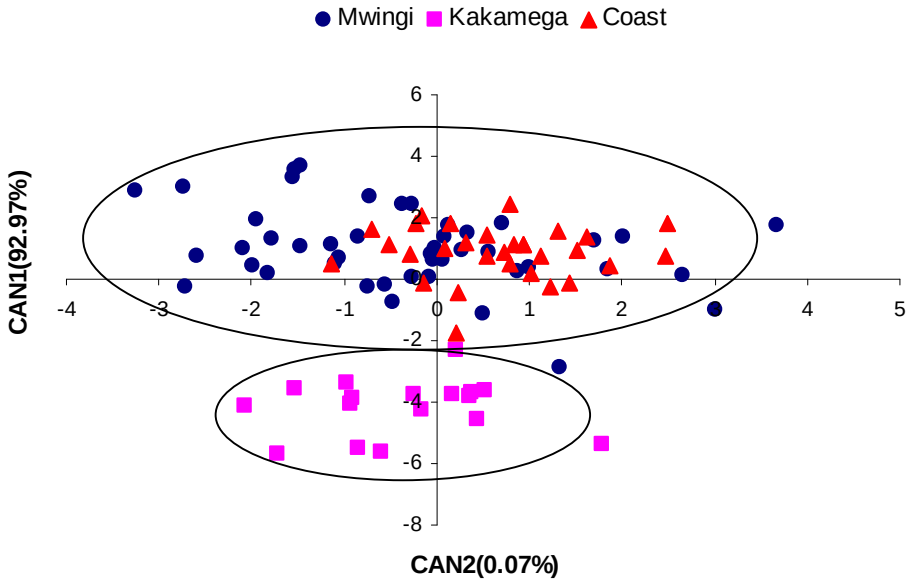

Fig. 9: Projection of wing vein data on first two canonical variates for *Hypotrigona gribodoi (Hg)* population collected from Kakamega, the population seems to be on the lower-side of the quadrant, Mwingi and Coast; Kakamega population separated from Mwingi –Coast populations. Mwingi-Coast population separated partially, with Mwingi population concentrated mainly on the left hand-side of the upper quadrant, while Coast samples are on the right-hand side of the upper quadrant.

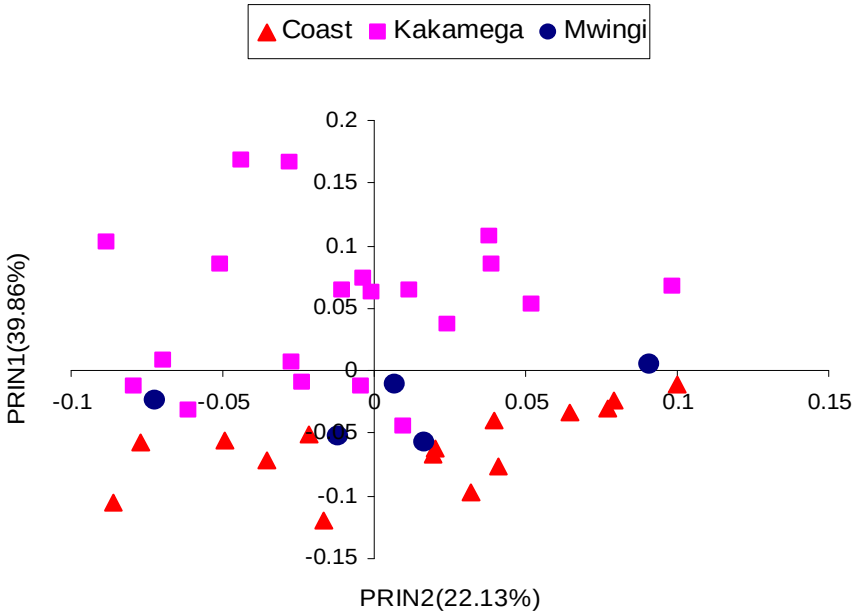

Fig. 10: Projection of wing vein data on first two principle components for *Meliponula bocandei* population collected from Kakamega, Mwingi and Coast: There is no clear separation of the three populations. There is partial separation where Kakamega is on the upper side of the quadrant then Mwingi is lying mainly in the middle and lastly Coast samples further down on the lower hand side of the quadrant.

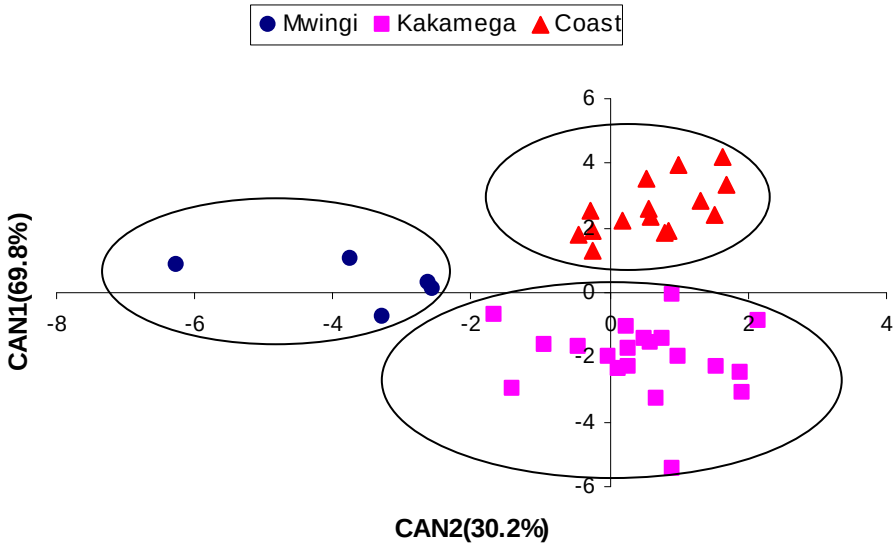

Fig. 11: Projection of wing vein data on first two canonical variates for *M. bocandei* population collected from Kakamega, Mwingi and Coast. The CV component separated the three populations into three distinct groups, with Kakamega on the lower side of the quadrant, Coast samples on the upper side and Mwingi o the left hand side of the upper side of the quadrant.

Table 3: Eigen values and weights of the first two principal components, computed from log-transformed wing data of *Hypotrigona gribodoi* species from three locations.

Variable	Weight	
	Prin1	Prin2
Proportion of variance (%)	58.27	22.58
Eigen values	0.017	0.006
WL	0.034	0.196
WW	0.043	0.156
V3	0.025	0.212
V4	0.069	0.414
V5	0.039	0.091
V6	0.023	0.255
V7	0.990	-0.131
V8	0.036	0.258
StigL	0.077	0.681
StigW	0.035	0.315

Weights of the first Principle Component (PC) were all positive while the second PC had some negative values. WL-Wing Length, WW-Wing Width, V3=Radial vein (R); V4= Rs; V5=basal vein (M); V6= Basal –Cubital vein (M+Cu); V7=Cu- V and V8 =V (anal vein).

Table 4: Eigen values and weight of the first and second principle components computed from log-transformed wing and leg data of *M. bocandei* species from three locations. Note that all values of Prin 2 are positive.

Variable	Eigen vectors	
	Prin1	Prin2
Proportion of variance (%)	39.86	22.13
Eigenvalues	0.005	0.0027
WL	0.080	0.155
WW	0.136	0.244
V3	0.035	0.108
V4	-0.062	0.574
V5	0.177	0.098
V6	0.013	0.143
V7	0.477	0.470
V8	0.042	0.188
StigL	-0.842	0.327
StigW	0.023	0.427

WL-Wing Length, WW-Wing Width, V3=Radial vein (R); V4= Rs; V5=basal vein (M); V6= Basal –Cubital vein (M+Cu); V7=Cu- V and V8 =V (anal vein).

Table 5: Raw, standardized and total canonical structure coefficient for canonical variate analysis on log transformed wing data for the *H. gribodoi* from (Mwingi, Kakamega and Coast).

Variable	CV1 coefficient			CV2 Coefficients		
	Raw	Standardized	Total CS	Raw	Standardized	Total CS
WL	43.42	0.94	0.82	-22.02	-0.48	-0.38
WW	-14.57	-0.3	0.59	9.47	-0.19	-0.19
V3	-30	-0.74	0.64	-53.33	-1.32	-0.66
V4	-0.48	-0.02	0.48	2.59	0.13	0.07
V5	-19.99	-0.43	0.24	-1.49	-0.03	-0.09
V6	28.44	0.84	0.9	12.27	0.36	-0.04
V7	2.06	0.27	0.18	1.7	0.22	0.19
V8	42.49	1.23	0.91	24.56	0.71	-0.18
Stig L	2.59	0.16	0.56	-2.09	-0.13	-0.27
Stig W	1.76	0.08	0.31	8.7	0.41	0.35

WL-Wing Length, WW-Wing Width, V3=Radial vein (R); V4= Rs; V5=basal vein (M); V6= Basal –Cubital vein (M+Cu); V7=Cu- V and V8 =V (anal vein).

Table 6: Raw, standardized and total canonical structure coefficient for canonical variate analysis on log transformed wing data for the *M. bocandei* from (Mwingi, Kakamega and Coast)

Variable	CV1 coefficient			CV2 coefficient		
	Raw	Standardized	Total	Raw	Standardized	Total
WL	-32.75	-0.51	-0.27	-60.59	-0.95	-0.08
WW	-16.42	-0.34	-0.36	-3.91	-0.08	-0.002
V3	25.14	0.4	-0.13	13.43	0.21	-0.11
V4	7.82	0.35	0.32	8.63	0.38	0.15
V5	-39.65	-1.01	-0.62	-1.34	-0.03	0.153
V6	64.9	1.07	0.32	140.54	2.33	0.3
V7	-2.38	-0.124	-0.46	14.88	0.78	0.14
V8	12.37	0.246	0.26	-97.92	-1.94	-0.32
Stig L	19.13	1.24	0.78	-5.28	-0.34	-0.12
Stig W	-1.76	-0.06	0.05	-9.8	-0.37	-0.4

WL-Wing Length, WW-Wing Width, V3=Radial vein (R); V4= Rs; V5=basal vein (M); V6= Basal –Cubital vein (M+Cu); V7=Cu- V and V8 =V (anal vein).

Table 7: Mahalanobis squared distance (D^2) between *H. gribodoi* species of the three locations.

Location	Coast	Kakamega	Mwingi
Coast	0		
Kakamega	21.3	0	
Mwingi	2	23.15	0

Table 8: Mahalanobis squared distance (D^2) between *M. bocandei* of the three locations.

Location	Coast	Kakamega	Mwingi
Coast	0		
Kakamega	13.69	0	
Mwingi	6.77	6.85	0

Table 9: Mean linear measurements and ratio calculations of leg measurements for *Hypotrigona gribodoi* populations sampled from three locations (Kakamega, Coasts and Mwingi).

Location	Mean length (mm)								Mean Ration (±SE)	
	WL	WW	TL	TW	FL	BAW	WWWL	TLWL	V6V3	TLV3
Coast	2.87±0.01a	1.2±0.01a	1.01±0.01a*	0.36±0.00a	0.8±0.01a	0.24±0.00a	2.38±0.00a*	2.86±0.00b*	1.37±0.00c	1.16±0.01b
Kakamega	2.65±0.04b	1.13±0.02b	0.86±0.02b	0.25±0.01b	0.7±0.01b	0.2±0.00b	2.33±0.00b	3.03±0.01a*	1.47±0.01a	1.28±0.01a
Mwingi	2.92±0.01a	1.21±0.01a*	0.98±0.02a	0.35±0.01a	0.78±0.00c	0.24±0.00a*	2.44±0 00a	3.03±0.01ba	1.41±0.00ab	1.25±0.02a*
F	48.53	16.11	10.91	57.72	71.11	40.61	8.2	3.58	15.16	5.828
df	2,90	2,90	2,90	2,90	2,90	2,90	2,90	2,90	2,90	2,90
p	<0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	>0.0005	>0.0319	<0.0001	0.0068

*Means in the same column followed by the same letters are not significantly different (p=0.05), using Student- Newman-Keuls (SNK) TEST. ANOVA performed on arcsine-transformed proportion values. WL-Wing Length, WW-Wing Width, V3=Radial vein (R); V4= Rs; V5=basal vein (M); V6= Basal–Cubital vein (M+Cu); V7=Cu-V and V8 =V (anal vein).

Table 10: Mean linear measurements and ratio calculations of leg measurements for *Meliponula bocandei* populations sampled from three locations (Kakamega, Coasts and Mwingi).

Location	Mean length (mm)						Mean Ration (±SE)			
	WL	WW	TL	TW	FL	BAW	WWWL	TLWL	V6V3	TLV3
Coast	5.27±0.06a	2.03±0.03a	1.75±0.04a	0.7±0.01a	1.52±0.02a	0.57±0.02a	2.56±0.00a	3.03±0.01a	1.67±0.00b*	1.52±0.01a
Kakamega	5.36±0.04a	2.10±0.02a	1.79±0.04a	0.7±0.01a	1.53±0.03a	0.56±0.01a	2.56±0.00a	3.03±0.01a	1.72±0.01ba*	1.52±0.01a
Mwingi	5.35±.08a	2.07±0.03a	1.71±0.07a	0.76±0.03a	1.54±0.03a	0.60±0.01a	2.56±0.00a	3.13±0.01a	1.75±0.01a	1.59±0.02a
F	1.22	2.25	0.52	1.54	0.04	0.77	1.52	0.52	5.27	0.66
df	2,37	2,37	2,37	2,37	2,37	2,37	2,36	2,36	2,36	2,36
P	0.3061	0.1196	0.6006	0.2276	0.959	0.4716	0.2323	0.5969	0.0098	0.5209

*Means in the same column followed by the same letters are not significantly different (p=0.05), using Student- Newman-Keuls (SNK) TEST. ANOVA performed on arcsine-transformed proportion values. WL-Wing Length, WW-Wing Width, V3=Radial vein (R); V4= Rs; V5=basal vein (M); V6= Basal–Cubital vein (M+Cu); V7=Cu-V and V8 =V (anal vein).

4.2 Discussion

4.2.1 DNA Barcoding

The purpose of the present study was to apply morpometric and molecular tools to identify and discriminate three species of stingless bees in Kenya. Universal barcode primers LCO1490 and HCO2198 were used to amplify 5' end of mitochondrial gene (COI) (Folmer *et al*., 1994). Hebert and Gregory (2005) stated that DNA barcoding is not intended to reconstruct phylogenetic relationship but instead focus on species delimitation and diagnostics. Sampling was done in different locations in Mwingi, Kakamega and Arabuko sokoke, for each species to maximize diversity within and between species so that overlap of intra- and interspecific divergence can be more accurately identified (Funk and Omland, 2003; Roe and Sperling, 2007). In addition, it has also been shown that broader sampling result into narrower gaps or partial overlap, between intra- and interspecific variation (Meyer and Paul, 2005).

Partial Cytochrome *c* oxidase 1 (COI) gene sequences were amplified from 92 samples of stingless bees using the universal primers LCO1490 and HCO2198. The 5'end of CO1 was chosen as the focal region because it is franked by two universal primers that work for a range of metazoans (Folmer *et al*., 1994) and has been shown to be most informative for species identification (Hebert *et al*., 2003a, b). Mitochondrial DNA (mtDNA) loci have long dominated the field of molecular systematics because of their maternal inheritance, limited recombination, rapid evolution, and the robustness of mtDNA against degradation, making them ideal markers for many species level questions (Avise *et al*., 1987). In addition the mtDNA is easy to isolate and sequence and the molecule evolves at a rate that is phylogenetically informative (Avise *et al*., 1987; Puorto *et al*., 2001). Agarose gel showed that all the sequences contained over 600 base pairs. This size of the amplified COI was in accordance to the standards required for barcoding (Herbert *et al*., 2003).

Data analysis clearly shows that base composition of the sequences was AT-rich as expected in insect mtDNA, AT composition was 78% while GC was 21.86% (Crozier and Crozier, 1993). ClustalX alignment showed areas of differences in nucleotide alignment between the three species. It is this difference in the nucleotide sequences that led into differentiating the three species. Earlier studies shows that nucleotide divergence is a primary criterion for delimiting species and detecting a cryptic species in initiatives such as DNA

taxonomy (Tautz *et al*., 2003; Monaghan *et al*., 2006) and DNA barcoding (Hebert *et al*., 2003a, b). Further analyses were done using MEGA 4. Neighbor joining tree (NJ) showed that there were identical gene sequences within species but the sequences differed between species; therefore the three species were separable by differences in base alignment. The high similarity in sequence alignment within species resulted into samples of each species clustering closely together; furthermore the samples separated depending on the regions from where they were collected. *Hypotrigona gribodoi* samples from Mwingi separated from samples from Coast and Kakamega with a distance of 0.036 and 0.033 respectively. The difference in the nucleotide sequence was probably due to the adaptation to the different environmental conditions since samples were collected from varying geographical areas, Kakamega is a high altitude compared to Mwingi and Coast (low altitude areas).

These results are comparable to Morphometic studies where Mahalanobis squared distance was 21.3 and 23.15 respectively. This shows that *H. gribodoi* from Mwingi are closely related to Kakamega than to the Coast samples. This also applied to the *M. bocandei* samples, where Mahalanobis squared distance between Kakamega and Coast was 13.69 while distance between Kakamega and Mwingi was 6.85. *Dactylurina schimdti* samples were collected from coast, this species is found only in the Coastal region of Kenya (Eardley, 2004). However, further studies by Gikungu *et al.*, 2006 showed that there might have been *D. schimidti* in Kakamega forest which disappeared due to habitat degradation. Analysis between groups shows that *H. gribodoi* is closely related to *D. schimidti (*D=0.084*)* than *M. bocandei (*D=0.111-0.118*)*. This is also clearly observed in the BOLD TaxonID tree where *M. bocandei* separates in one branch while *H. gribodoi and D. schimidti* were in one branch. The intraspecific divergence was low (<2%) compared to inter specific divergence (>8.4%). This is consistent with studies done by Meyer and Paulay, (2005) who states that the nucleotide diversity within species should be lower than between species.

The results imply the future utility of barcoding in the identification of stingless bees. A biologist unfamiliar with stingless bee species could, using the classic barcoding segment of COI, place any of the collected stingless bee specimens in their correct major clade. The generated barcode sequences with accession numbers (BOFAS001-BOFS90-08) will offer a very good chance for correct identification of the three stingless bees' species in the future.

4.2.2 Morphometrics

Morphometric analyses have proved to be very efficient and fast for identification of African honeybee (*A. mellifera*) (Francoy *et al*., 2006). Evolution of computerized morphometrics systems has led to identifying species of various insect groups using only one cell of the wing (Steinhage *et al* 2001; Tolfiski, 2004; Francoy *et al.*, 2006; Drauschke *et al.*, 2007; Mendes *et al*., 2007; Steinhage *et al*., 2007). Morphometrics studies of the three stingless bee species was therefore, considerably improved by use of computer softwares. The PC analysis shows that for *H. gribodoi* samples, all the first principle components were positive and within a range of 0.023- 099. Separation along the first component is usually associated with size, especially when the weights are positive (Julicoer and Masimman, 1960). Thus, *H gribodoi* populations were separated by size. The variables that contributed most were V7 (Cu-V) and stigma length (SL) contributing 0.99 and 0.76 respectively. The variables with least contribution to variation were V3 (R-Radial vein) and M-Cu; Medial cubital vein (V6). There is a significant difference between the Kakamega population and Mwingi-Coast population. This is clearly depicted in the canonical variate analysis. Populations from Kakamega were placed mainly on the lower side of the quadrant, Mwingi samples were on the upper-left–side of the quadrant while Coast population was in the upper–right–side of the quadrant. Strong positive standardized coefficient sores for variables WL (wing length), V6 (medial cubital vein) and V8 (V-anal vein) (0.94, 0.84 and 1.23) respectively, in the first canonical variate analysis indicate that these variables plays a major role in distinguishing *H. gribodoi* population from three localities.

On the other hand, *M. bocandei* populations were separated by shape. In this case there were some negative values in the first principle component while all values were positive in the second principle component ranging between 0.09-0.573. The variable contributing to the variation were V4 (Rs vein) and V7 (Cu-V vein) contributing 0.57 and 0.47 respectively. The three populations were significantly different, Kakamega population were placed in the lower quadrant while coast samples were on the right side of the upper quadrant, Mwingi samples were on the left quadrant.

Analysis of Mahalanobis distance between the centroids of the group revealed that Kakamega populations were significantly different from Mwingi and Coast populations (Table 5 and 6). Mahalanobis distance (D^2) can be used to assign an unidentified specimen to a specific group, the near it is the more related they are (Billah *et al*., 2005). The mean linear

measurements for *H. gribodoi* show that Femur length (FL) as the best discriminant than WW, WW, TL, TW and BAW. In the ration calculations V6V3 (M+Cu (Medial- Cubital vein/Radial vein) was the best for separating the *H. gribodoi* population. However the mean linear measurements could not discriminate the *M. bocandei* samples. V6V3 ratio was better in the separation of *M. bocandei* populations.

This morphological analysis when associated with molecular and behavioral data is useful in future studies that will improve our understanding of biology and evolution of stingless bees in Kenya. The present results support the use of wing morphometrics to retrieve relevant information about origins of a population (Dujardin *et al*., 2007).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Morphometric technique was able to differentiate the three stingless bee species based on the wing and leg measurements. However, application of DNA barcoding techniques speeded up the sorting and identification of specimens. *Hypotrigona gribodoi* and *Meliponula bocandei* species collected from the three regions are different genetically probably due to the adaptation to the different environmental conditions. Discrimination of species is important to farmers for breeding purposes and the production of quality honey.

5.2 Recommendations

This study recommends that both techniques should be incorporated to enhance accuracy and efficiency. In addition, morphological description of the stingless bee should be undertaken before any molecular work. Currently, the species are under described this should be given the first priority to maximize on more species discovery. More specimens should be collected from the different regions in Kenya and be described. Lastly, commercial breeding aspects of these bees should be enhanced to improve honey production.

CHAPTER SIX

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