THE ROLE OF BATS IN THE BIOLOGICAL CONTROL OF PESTS FROM MACADAMIA ORCHARDS IN LIMPOPO PROVINCE, SOUTH AFRICA

BY

EMMANUEL MATAMBA

FEBRUARY 2015

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THE ROLE OF BATS IN THE BIOLOGICAL CONTROL OF PESTS FROM MACADAMIA ORCHARDS IN LIMPOPO PROVINCE, SOUTH AFRICA

By

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Submitted in partial fulfilment for the Masters of Science

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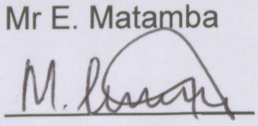
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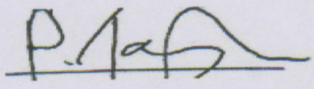
STATEMENT OF ORIGINALITY

I, MATAMBA EMMANUEL hereby declare that the dissertation for the Masters of Science in Zoology degree at the University of Venda, hereby submitted by me, has not been submitted previously for a degree at this or any other university, that it is my own work in design and in execution, and that all reference material contained therein has been duly acknowledged

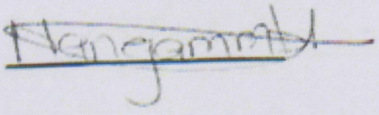
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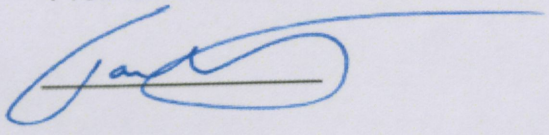
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DEDICATION

This thesis is dedicated to Mulisa Matamba (my daughter) who by the time I wanted to quit University of Venda she came to life and needed my support and I had to accept Prof Taylor's proposal to do this project under the supervision of Dr Nangammbi. They allowed me to take a very good opportunity by doing this project with the collaboration of the University of Copenhagen, Denmark.

This thesis is also dedicated to all other people who helped in the success of this project.

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ABSTRACT

Stinkbugs (Heteroptera: Hemiptera: Pentatomidae) which damage macadamia are important pests for many crops including macadamia, feeding mostly on seeds and immature fruits. DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species. DNA barcoding of agricultural pests is a high priority as it allows rapid and accurate identification of pests. Too few of these pest insects have been DNA-barcoded and this leads to a problem, e.g. when identifying pests or possible biological control agents. Insectivorous bats are believed to feed on pest insects in agricultural lands. However, a study to see if bats do eat stinkbugs and how they can be used to control these pests has never been undertaken in South Africa.

This study focuses on 10 bug species which includes some that are known to be agricultural pests. Some of the stinkbugs included in the study belong to families other than Pentatomidae and, only *Nezara viridula* has been listed on GenBank. Using the barcoding region (mitochondrial cytochrome oxidase I control region) 658 bp was sequenced, and analysed in relation to available hemipteran sequences on GenBank, with the aim to DNA barcode the pests and determine the phylogenetic relationship of stinkbugs in South Africa. The results indicate that there is still a lot to be done in South Africa to have a complete record of the pests.

In connection with insect sequences available on GenBank and BOLD, the newly acquired sequences allowed to probe the diet of bats, using next generation sequencing (NGS) of fecal pellets of six species targeting only 157 bp of the COI region, and NGS data from bat fecal pellets matched the known diet of six South African bat species based on previous microscopic dietary studies but provide greater taxonomic precision of prey items eaten down to family, genus and species level though most of pests in South Africa were not found. All three foraging groups from clutter-feeding bats, clutter edge bats and open-air feeders feed on pest *Nezara viridula* stinkbug which is not what was originally expected given that stinkbugs are not very mobile and spend much of their time resting on branches.

Keywords; Macadamia, DNA Barcoding, Biological control, next generation sequencing

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
Bst DNA polymerase	*Bacillus stearothermophilus* DNA polymerase
rRNA	ribosomal Ribonucleic Acid
MtDNA	Mitochondrial DNA
COI	Cytochrome C Oxidase I
PCR	Polymerase Chain Reaction
IPM	Integrated Pest Management
ml	Millilitre
µl	Microliter
^0C	Degrees Centigrade
rpm	Revolutions per minute
TAE	Tris-Acetate EDTA
%	Percentage
NaCl	Sodium Chloride
$MgCl_2$	Magnesium Chloride
mA	Mill amperes
V	Volts
W	Watts
UV	Ultra-Violet

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µM	Micro Molar
mol	Molar
dNTP's	Deoxynucleoside Triphosphate
ddH_2O	Double Distilled Water
bp	Base pairs
g	grams
mg	Milli grams
xg	Times gravity
BOLD	Barcoding of Life Database
BSA	Bovine Serum Albumin
NJ	Neighbour Joining
InPe	Index Primer Paired End
PAUP	Phylogenetic Analysis Using Parsimony
MEGA	Molecular Evolutionary Genetics Analysis

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CHAPTER 1. GENERAL INTRODUCTION

1.1. BACKGROUND INFORMATION

Macadamia, which belongs to the family Proteaceae, is grown for its edible kernel. It is a dark green spreading semi-hard wood evergreen tree (Gitonga *et al.* 2010). Macadamia nuts are very important to human health, since they can reduce lipid/lipoprotein cardiovascular disease risk factors if they are included in a heart-healthy dietary pattern (Griel *et al.* 2008). Macadamia nut consumption favourably modifies the plasma lipid profile of hypercholesterolemic men when included as part of a healthy diet although it is high in fat (Garg *et al.* 2003). Consumption of diet high in mono-unsaturated fat derived from macadamia nuts had potentially beneficial effects on lowering cholesterol levels. Coupled with the palatability of the macadamia, this leads to a suggestion that macadamias contribute to a satisfying and healthy diet (Garg *et al.* 2003; Curb *et al.* 2000).

Since the discovery of their importance to human health, the macadamia industry in South Africa is amongst the top three macadamia producers in the world (Groenewald 1999). However humans compete with a multitude of pest species for food, fibre and timber among other things, although natural predators greatly reduce the densities of many of these pests (Daily 1997; Cleveland *et al.* 2006); this also applies in macadamia orchards.

Universally, stinkbugs (Hemiptera: Pentatomidae and Coreidae) have been identified as the major pest of macadamias; their damages in the macadamia and avocado industries amounts up to R50 million annually in South Africa (Schoeman 2013). The feeding damage of the stinkbugs (specifically the southern green stinkbug, *Nezara viridula*), on nut crops has been known since 1917 and has been recorded on macadamia since 1964 (Mitchell *et al.* 1965).

In South Africa, pentatomid and coreid stinkbugs have been recorded as pests in macadamia orchards (Van den Berg *et al.* 1999, Schoeman 2013). Other important pests recorded in macadamia orchards include moths (Lepidoptera: Tortricidae), which are also responsible for nut damage (Schoeman 2009). Although most of the insect damage has been recorded, some insect nut damage is not recorded, because most of the nuts that are damaged after early thinning fall and are considered litter when farmers start clearing as a preparation of harvest (Schoeman 2009).

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As a result of these damages; growers attempted spraying programmes which produced exceptional results at the beginning but later on it was reported that there was proliferation of secondary pests. And from that and a demand of environmentally and socially sustainable fruit production from importing countries growers are forced to use approaches which will not harm the environment and still produce good crop protection against pests (Schoeman 2013).

This led Schoeman (2013) to recommend implementing integrated pest management (IPM), which is the selection, integration and implementation of pest control which matches economic and ecological needs (Bottrell 1979).

Attention to recent studies has been paid, particularly to biological control of agricultural pests by both diurnal (birds) and nocturnal (bats) flying predators (Maas *et al.* 2015). Bats have been recorded eating stinkbugs (Hemiptera: Pentatomidae) and moths (Feldhamer *et al.* 2009; Bohmann *et al.* 2011; Kunz *et al.* 2011; Maas *et al.* 2013; Taylor *et al.* 2013; Valdez and Cryan 2013). Bats are the second largest order of mammals, with over 1,100 species that have diverse diets, including blood, fish, pollen, fruit, nectar, and insects (Rolfe 2011; Schnitzler and Kalko 2001).

In this project, insects' specimens were collected in macadamia orchards and their Deoxyribose Nucleic Acid (DNA) that were extracted from their legs was sequenced to obtain a reference library of DNA barcode sequences to find out whether bats can be used as biological control agents of these pest insects. To achieve this objective fresh bat faecal pellets were aslo collected and PCR amplification of minibarcodes for Illumina sequencing was used to identify the insect DNA extracted from these pellets and to compare them with both our collected insect sequences as well as published sequences using a bioinformatics approach.

1.1.1. OVERVIEW OF THE MACADAMIA INDUSTRY

The agriculturally important macadamia is either of two species of macadamia indigenous to the coastal rain forest of southern Queensland and northern New South Wales in Australia, which are cultivated for their large seeds with edible kernels that are the macadamia nuts (Hamilton and Storey 1956) and those species are *Macadamia ternifolia* F. von Mueller, and *Macadamia tetraphylla* L. Johnson and in some instances their crossbreed (Groenewald 1999). F. von Mueller described it botanically in 1858, naming *it Macadamia ternifolia* in

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honour of Dr John Macadam, President of the Philosophical Society, Victoria, Australia (Hamilton and Storey 1956).

The smooth-shell *Macadamia ternifolia* is the more tropical species, occupying a range habitat from central to southern Queensland. The rough-shell *Macadamia tetraphylla* grows naturally only in the northern coastal section of New South Wales and in an adjacent area of the southernmost part of Queensland. Macadamia is believed to have been imported to Hawaiian Islands, in the United States of America around the 1880's (Hamilton and Storey 1956).

Macadamia nut production is possibly high on deep well-drained soils with rainfall in excess of 1524 millimeters per year. It is a relatively hardy drought-tolerant tree, once it has become established (Hamilton and Storey 1956). The trees require a hot subtropical climate without much humidity, in South Africa KwaZulu-Natal, Mpumalanga and Limpopo are ideal areas (Morokolo 2011).

When matured, macadamia nuts fall from their trees during harvesting and are then picked up and packed by hand. They characteristically ripen over a period of several months because of intermittent blossoming and must be harvested rather often, especially during rainy weather (Hamilton and Storey 1956).

In South Africa the macadamia industry provides employment for more than 4500 farm workers and about 1500 factory workers, and since production is expected to double within the next five to seven years, this suggests the macadamia industry is still growing. There is no regulation or restriction in the marketing of macadamia nuts in South Africa. Though more than 95 % of annual production is shipped to international markets, there are buyers of macadamia inside South Africa (Morokolo 2011).

The kernel is the main product from the macadamia nut tree. After harvesting, the husks covering the nuts are removed. The nuts are fried, the shells are cracked, and the kernels are removed to be oil-roasted or dry-roasted (Morokolo 2011).

1.1.2. PEST INSECTS ON MACADAMIA NUTS

Levubu, which is the study area, is the biggest production area of macadamias in South Africa; however the future of the industry is affected by serious constraints. Macadamia are

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attacked by indigenous pests which include moth species, stinkbugs and thrip species (Schoeman 2009).

The most important pests of macadamias are true bugs. The true bugs (Insecta: Heteroptera) have been recorded to represent the largest group of hemimetabolous insects, with more than 42,000 described species in over 5800 genera and 140 families (Park *et al.* 2011; Henry 2009). The order includes many economically important plant pests, animal disease vectors and predators employed in biological control (Schwartz and Foottit 1998). One of the most important groups in Heteroptera is the suborder Pentatomomorpha, which was recorded as the second largest group, ranging between 12 500 to 15 000 species worldwide. However phylogenetic relationships within members of this group are not well documented (Li *et al.* 2005) and it is dominating factor in the macadamia industry in South Africa (Schoeman 2009).

The green stinkbug *N. viridula* (L.) and more than thirty other stinkbug species are the most important pests that feed on macadamia in South Africa and these stinkbugs are important pests of many crops, feeding mostly on seeds and immature fruits (Van den Berg 1995). Southern green stinkbug *N. viridula*, (Hemiptera: Pentatomidae) is the most important pentatomid pest of agriculture all over the world (Todd 1989), including macadamia nuts where they cause pitting to kernels by feeding on them (Madsen 1950; Jones *et al.* 2001; Wright *et al.* 2007).

The feeding of *N. viridula* actually increases losses in the macadamia industry because they also cause premature drop, resulting in immaturity, and also assist in the introduction of pathogens causing mouldy nuts, both of which result in rejection at the processor. Several growers with large acreage have begun applying insecticides to control *N. viridula* when large numbers of the stinkbugs are observed on broadleaf weeds in the orchard (Golden *et al.* 2006). Ethiopia is believed to be the native geographical range of *N. viridula*, although some authors still confuse it with Southern Europe and parts of the Mediterranean region (Hokkanen 1986; Jones 1988) and other species in its genus occur in Africa and Asia and closely related genera occur in South America, Asia and Australia (Freeman 1940).

In South African macadamia orchards, the two spotted bug *Bathycoelia natalicola* is the most abundant species because this bug comprises half of the total number of Pentatomidae insects. Its abundances were followed by *Nezara* species (*N. pallidoconspersa* and *N.*

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prunasis). *B. natalicola* has been recorded as a primary pest of macadamia and has seen to cause the highest damage as compared to other pests (Schoeman 2009).

With the presence of *B. natalicola, N. viridula, N. pallidoconspersa, N. prunasis* and many other stinkbugs in macadamia these stinkbugs becomes the most important pests of macadamia in South Africa (Van den Berg 1995; Schoeman 2009; Taylor *et al.* 2013; Schoeman 2013). The coconut bug, *Pseudotheraptus wayi* (Heteroptera: Coreidae) has also been found in macadamia orchards. Though the damage caused by *P. wayi* on macadamia has not been seen as a threat it is a dominant pest in other subtropical orchards such as avocados, mangoes and guavas (Schoeman 2009). It is important to note that the Heteroptera complex does not damage any harvested aborted physiologically mature nuts in South African and hence there is no need to worry about fallen nuts.

Moths of the Tortricidae are also other important macadamia pests which are also serious pests of macadamias. The false codling moth *Thaumatotibia leucotreta* and the macadamia nut borer *Thaumatotibia batracopa* (Lepitidoptera: Tortricidae) species has been previously identified as primary pest in South African macadamia nuts, whereas the Litchi moth *Cryptoplebia peltastica* (Meyrick) has been classified as sporadic pest (Schoeman 2009).

The economic importance of *Thaumatotibia leucotreta* was recognised in the South African citrus industry citrus since the beginning of the century. The moth was first described as a pest of citrus in Natal (Fuller 1901). It was previously known as *Cryptoplebia leucotreta* and has only recently been classified as *Thaumatotibia leucotreta* (Komai 1999). *C. leucotreta* has a very wide range of host plants (Schwartz 1981). *Thaumatotibia leucotreta* is known to be hosted by more than 70 plants including citrus, avocado, litchi, macadamia and many more agricultural crops. *Thaumatotibia leucotreta* damages the macadamia nuts by larval feeding on maturing kernel (Jones 1995).

1.1.3. DIVERSITY OF BATS

Megachiroptera, which was classified as fruit eating bats and Microchiroptera, which was classified as insect-eating; were the suborders which were previously recognized in the order Chiroptera. These two suborders are still widely used outside of scientific circles (Hutcheon and Kirsch 2006; Monadjem *et al.* 2010; Kruger *et al.* 2010).

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However after molecular evidence showed a close relationship between species of suborder Megachiroptera and some families which were previously classified as belonging to the suborder Microchiroptera, these suborder names were revised and Pteropodiformes is currently the name of the order containing fruit eating bats along with some insectivorous bats (includes Craseonycteridae, Hipposideridae, Megadermatidae, Rhinolophidae, Rhinopomatidae, and Pteropodidae) while Vespertilioniformes is the name for the remaining families of bats (including Emballonuridae, Nycteridae, Vespertilionidae and Molossidae) (Hutcheon and Kirsch 2006).

Insectivorous bats in Africa predominantly feed on insects. Some glean insects from vegetation and water in cluttered forests while others feed in open space above forests, grasslands, and agricultural landscapes while frugivorous bats include species that feed in fruits, nectar and pollen (Kunz *et al.* 2011).

1.1.4. FORAGING ECOLOGY OF BATS

Diets can differ between species, but also within species. This intraspecific variation in diet results from intrinsic factors, such as increased energetic and nutritional needs for hibernation, migration, growth, and especially reproduction (Murray and Kurta 2002). Studies have shown that many bat species prefer certain foraging habitat characteristics and hence availability of preferred habitat influences species abundance (Duchamp *et al.* 2004).

The diets of bats also differ greatly: Frugivorous and nectivorous bats can feed on the fruits, leaves, flowers and nectar of a wide range of indigenous trees. Carnivorous bats feed on smaller vertebrates such as fish, frogs, mice, birds, and even other bats. The other 70% of bat species worldwide are insectivorous and hence they eat insects (Jones and Rydell 2003; Monadjem *et al.* 2010)

The diet of an insectivorous bat is necessarily dependent on the types of insects that are available. The abundance of insects is highly variable in space and time, and it can be expected that the diet of an insectivorous species vary with the seasons (Aldridge and Rautenbach 1987; Murray and Kurta 2002).

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A variety of local bat species forage in macadamia orchards. Seasonal variation of foraging activity of bats in macadamia orchards corresponds with the times of the year when major infestations of pest insects are known to occur (Taylor *et al.* 2011; Taylor *et al.* 2013).

Insectivorous bats include open-air foragers that capture their prey while flying high above the ground. As this is far from vegetation, it helps them avoid problems with clutter echoes and obstacles. Clutter-edge foragers hunt for insects near the edges of forests. Clutter foragers search for insects and capture in highly cluttered space close to the ground or vegetation, distinguishing insect echoes buried in clutter echoes, while at the same time locating their exact spatial position to avoid collision with the clutter (Monadjem *et al.* 2010)

1.1.5. ECONOMIC IMPORTANCE OF BATS

Ecosystem services are defined as the benefits to human welfare provided by organisms interacting in ecosystems (Klein *et al.* 2007; Kunz *et al.* 2011).

1.1.5.1. ECOSYSTEM SERVICES OFFERED BY FRUGIVOROUS BATS

Pollination by wild animals is a key ecosystem service because it is important for sexual reproduction of many crops (Klein *et al.* 2007). Two primary families of bats (Pteropodidae in the Old World and Phyllostomidae in the New World) play an important role as pollinators and seed dispersers in tropical and subtropical habitats throughout the world (Kunz *et al.* 2011).

Of the Pteropodidae, only 15 species in six genera are morphologically specialized nectar-feeders other members of this family are primarily fruit-eaters and of the Phyllostomidae, about 38 species in 16 genera are specialized nectar-feeders while 90 species in 22 genera are primarily frugivorous. However other species from other genera within these two families are opportunistic flower visitors. This is so because to feed on nectar and pollen requires relatively specialized morphology (Kunz *et al.* 2011).

Pollination and seed dispersal are mutualistic population interactions in which plants provide a nutritional reward in the form of nectar, pollen, and fruit pulp, to animals for beneficial services which are pollen and seed dispersal (Kunz *et al.* 2011).

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Dietary accumulation and metabolic capacity increases at higher positions in the food chain, and insectivorous bats are likely to show the consequences of pollutants before organisms at lower trophic levels such as herbivorous insects or birds. And since insectivorous bats occupy higher trophic levels, their taxonomy is mostly stable, at least at the species level they would be excellent bio-indicators owing to the relationship between contaminant and/or environmental disturbance and trophic levels (Jones *et al.* 2009).

Bats are voracious predators of nocturnal insects, including many crop and forest pests, hence insectivorous bats are of ecological and economical importance to farmers and society because they are seen reducing the costs of pesticide use (Cleveland *et al.* 2006, Boyles *et al.* 2011).

Bats also assist in the consumption of specific agricultural pests including June beetles (Scarabidae), click beetles (Elateridae), leafhoppers (Cicadelidae), planthoppers (Delphacidae), the spotted cucumber beetle, (*Diabrotica undecimpunctata*, Chrysomelidae), the Asiatic oak weevil (*Cyrtepistomus castaneus*, Curculionidae), and the green stinkbug (*Acrosternum hilare*, Pentatomidae) (Kunz *et al.* 2011).

1.1.6. BATS SPECIES INCLUDED IN THIS STUDY

The clutter foragers (Nycteridae and Hipposideridae); open air foragers (Molossidae); and clutter-edge foragers (Vespertilionidae) are included in this study so as to cover all groups of foragers.

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1.1.6.1. *Nycteris thebaica* (Chiroptera: Nycteridae)



Figure 1: *N. thebaica*, picture adapted from Monadjem *et al.* (2010).

Also known as Egyptian slit-faced bat, this is a small bat with a mass of around 10 g which is identified by its facial slit covering the noseleaf. It produces low-intensity, multi-harmonic Low–Duty cycle Frequency–Modulated (LD–FM) echolocation calls with a high main peak frequency and short duration. Although it is absent from much of the plateau grasslands of South Africa, this species has been recorded widely throughout the entire region, from all the southern African countries except Lesotho (Monadjem *et al.* 2010).

N. thebaica roosts during the day in a variety of shelters including caves, aardvark burrows, culverts under roads, and the trunks of large trees. It forages by flying low above the ground, listening for prey scurrying on the surface (Monadjem *et al.* 2010).

The diet of this bat has been studied in South Africa before using microscopic analyses of faecal pellets. In Levubu, there were significant differences in diet between months, with five orders of insects being presented as dominant including bugs (Taylor *et al.* 2011). In another study the bat has been reported to feed on nine insect orders based on the culled parts visible in microsocopic analysis (Taylor *et al.* 2012). As already explained above, the foraging strategy of this bat which is expected to feed on stinkbugs makes it relevant for inclusion in this study (Taylor *et al.* 2011; 2012).

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1.1.6.2. *Scotophilus dinganii* (Chiroptera: Vespertilionidae)



Figure 2: *S. dinganii*, picture adapted from Monadjem *et al.* (2010).

Also known as a yellow house bat, this is a medium-sized bat with a mass of around 26 g which is identified by having sleek fur, a long narrow tragus, and yellow under-parts; though similar to *Scotophilus viridis,* its larger size differentiates it. This species produces LD-FM echolocation calls with an intermediate peak echolocation frequency, narrow bandwidth, and intermediate duration. Like *N. thebiaca* it is absent from the plateau grassland and Karoo of South Africa and the Kalahari, but has been recorded widespread the southern African countries (Monadjem *et al.* 2010).

It roosts during the day in a variety of shelters including holes in trees and roofs of houses; it has the habit of roosting in fabricated structures. This bat is a clutter-edge forager. Its diet is mainly dominated by medium-sized Coleoptera, although it may also include Hemiptera, Hymenoptera, Isoptera and Diptera (Monadjem *et al.* 2010).

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1.1.6.3. *Pipistrellus hesperidus* (Chiroptera: Vespertilionidae)



Figure 3: *P. hesperidus,* picture adapted from Monadjem *et al.* (2010).

*P. hesperidus*is a small bat with a mass of around 6 g and is very similar in appearance to the slightly smaller *Pipistrellus rusticus*. Although it is difficult to distinguish in the field from other *Pipistrellus, Hypsugo* or *Neoromicia* species, its wings are dark brown to black and the face is plain, without any noseleafs. This species produces LD-FM echolocation calls with an intermediate peak echolocation frequency, broad bandwidth, and short duration, and occurs widely in the eastern parts of the southern African region (Monadjem *et al.* 2010).

Its roosting habits are poorly known in southern Africa, but it is a clutter-edge forager, with a diet that includes Coleoptera, Hemiptera, Diptera and Lepidoptera (Schoeman 2006; Monadjem *et al.* 2010).

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1.1.6.4. *Mops midas* (Chiroptera: Molossidae)



Figure 4: *M. midas*, the picture was adapted from Monadjem *et al.* (2010).

Known as Midas free-tailed bat, *M. midas* is a large-sized bat with a mass of around 45 g; a sparse-haired band across the shoulders separates *Mops* from *Tadarida* and most *Chaerephon* species. Its large size should prevent confusion with any other *Mops* species except two other *Mops* species (*M. congicus and M. niangarae*); there are also large species but have never been recorded south of the equator. This species produces Low-Duty cycle Quasi-Constant-Frequency (LD-QCF) and Low-Duty cycle Constant-Frequency (LD-CF) echolocation calls and it is locally abundant in the eastern parts of the southern African region (Monadjem *et al.* 2010).

It is an open-air forager based on very limited data from a few stomach contents, it was suggested that it feeds predominantly on Coleoptera (Archer 1977). It was first recorded as a large, tropical species of free- tailed bat, in South Africa at Kruger National Park in 1971. It was identified as a relatively rare inhabitant of open woodland and savannah (Pienaar 1972).

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1.1.6.5. *Chaerephon pumilus* (Chiroptera: Molossidae)



Figure 5: *C. pumilus* , picture adapted from Monadjem *et al.* (2010).

Also known as the little free-tailed bat, *C. pumilus* is a small bat with a mass of around 10 g. This bat is usually differentiated from other free tailed bats by its smaller size. This species produces LD-QCF calls with a low peak frequency, narrow bandwidth, and long duration (Monadjem *et al.* 2010). Variation in colour, size and echolocation across its wide distributional range and the occurance area light-winged form in north-eastern Africa and a dark form in southern Africa prompted Jacobs *et al.* (2004) to investigate if there may be cryptic species present; however genetic analysis suggested that there is only one species. But later stages revealed cryptic species within this taxon across its wide range in Africa and the Indian Ocean islands (Goodman *et al.* 2010)

Chaerephon pumilus is widespread and abundant in the eastern parts of the southern African region; it is an open-air forager, feeding on Coleoptera, Hemiptera, Lepidoptera, Hymenoptera and Diptera (Aldridge and Rautenbach 1987; Monadjem *et al.* 2010).

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1.1.6.6. *Hipposideros caffer* (Chiroptera: Hipposideridae)



Figure 6: *H. caffer*, picture adapted from Monadjem *et al.* (2010).

Also known as Sundevall's leaf-nosed bat, *H. caffer* is a small bat with a mass around 8 g. It's simple, elliptical noseleaf distinguishes, these genus from all other Southern African bats. *H. caffer* produces High–Duty cycle Constant–Frequency (HD–CF) calls with a high peak frequency and intermediate duration. Though it is absent from much of the southern and a central part of the region, the species is widely distributed throughout southern Africa (Monadjem *et al.* 2010).

Hipposideros caffer roosts in a wide range of caves, sinkholes and cavities, including anthropogenic ones such as mines and culverts (Cotterill 2001, Monadjem *et al.* 2010). It is a clutter forager, with the diet consisting predominantly of Lepidoptera, and smaller quantities of Trichoptera and Coleoptera (Schoeman 2006; Monadjem *et al.* 2010).

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1.2. PROBLEM STATEMENT AND RATIONALE FOR THE STUDY

Agricultural pest insects (mainly stinkbugs) are economically important since they cause losses to the macadamia industry approximating an average of R50 million per year in South Africa. This led to the suggestion of IPM (Integrated Pest Management) of these pests; however none of the biological pest control has been identified in South African macadamia orchards (Schoeman 2013). The initial work (Taylor *et al.* 2013) indicated that bats do eat the green stinkbug (*N. viridula*) hence bats are believed to play a role in pest management in stinkbugs, only Bohmann *et al.* (2011) has studied the role of bats in Africa and no work has been documented in Macadamia orchards in Africa. It is important to note that the economic importance of insectivorous bats in agriculture has been studied in many countries outside of Africa (Agrawal 2001; Miner *et al.* 2005; Cleveland *et al.* 2006; Feldhamer *et al.* 2009; Jones *et al.* 2009; Boyles *et al.* 2011; Kunz *et al.* 2011; Maas *et al.* 2013). All these studies indicated that bats do play an important role in pest control and no one knows if this is the case in South African macadamia orchards, given that the pests are different.

It is also important to note that some of the pest insects in South Africa have never been recorded as pests elsewhere in agricultural lands. For example, Schoeman (2013) identified *B. natalicola* and *N. pallidoconspersa* as macadamia pests which are endemic to South Africa. To understand the role of bats in biological control of these pests there is a need to know about these pests. Grazia *et al.* (2008) determined relationships among the Pentatomoidae family using morphology and molecular (18S rRNA, 16S rRNA, 28S rRNA, and COI gene regions) data. However, in their research they did not include any of the South African stinkbugs and it appears that almost all South African agricultural pests and non-pests stinkbugs have not been identified using DNA barcode. Inorder to find the predators it is important to have reference sequences and in case of South African stinkbugs it is difficult to find biological control agents since none of them have their sequences deposited in online databases for reference.

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1.3. RESEARCH QUESTIONS

1. What do bats in South African macadamia orchards feed on?
2. How does the diet of six species of bats from a macadamia agro-ecosystem compare with published literature for these species and does it include pest species of macadamia including pest stinkbugs of the Pentatomidae and Coreidae complex and pest moths of the Torticidae complex?
3. Do different species of bats having different foraging strategies feed on different pest insects having different flight behaviour?
4. Is DNA barcoding an effective tool to identify both pest and non-pest species of stinkbugs occurring in macadamias?
5. Can the barcode gene (COI) be used as a phylogenetic marker to determine the relationships between pest and non-pest species of stinkbugs occurring in macadamias?

1.4. AIMS AND OBJECTIVES OF THE STUDY

1. To collect, identify by aid of a hemipteran specialist (Dr M. Stiller, ARC) and sequence using the COI gene, a range of hemipteran species from local macadamia orchards in order to test the value of a barcoding approach for rapid identification of this group of insects, as well as for clarifying phylogenetic relationships between members of three families within the Pentatomoidea.

2. To collect bat pellets of six local bats (identified by Prof Taylor and Mr Steyn) to accurately determine their diets using a short COI gene fragment amplified using tagged primers for Next Generation Sequencing.

3. To accurately determine if different species of bats with different foraging strategies, feed on different pest insects having different flight behaviour by comparing the diets after Next Generation Sequencing.

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1.5. RESEARCH HYPOTHESES

1. Sequence data for the COI gene should allow correct identification of the hemipteran pest and non-pest species collected from macadamia orchards in this study, making DNA barcoding a useful tool for rapid identification of this major group of pests of the macadamia industry.
2. Sequence data for the COI gene from hemipteran species and genera should provide a useful phylogenetic marker to allow an improved phylogenetic analysis of southern African bugs (Hemiptera) and in particular to test the monophyly of the two "stinkbug" families, Pentatomidae and Coreidae.
3. Next Generation Sequencing data from bat fecal pellets should corroborate the known diet of six South African bat species based on previous microscopic dietary studies but provide greater taxonomic precision of prey items eaten down to family, genus and species level depending on availability of reference sequences on GenBank and BOLD.
4. The slow-flying stink-bugs should be more prevalent in the diet of clutter-feeding bats (such as *N. thebaica* and *H. caffer*) and clutter edge bats (such as *P. hesperidus*and *S. dinganii*) and less prevalent in open-air feeders like *M. midas* and *C. pumilus* .
5. Since moths are known to be prevalent in the diet of most of the bats included in this study and previous researches has shown that the moth macadamia nut borer peaks in December and January, this moth is predicted to be present only in bat fecal pellets collected in December and January.

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1.6. STUDY LOCATION

The study area is located in the southern foothills of the Soutpansberg Range, 20km east of the town of Makhado (previously, Louis Trichardt), encompassing the Levuvhu Valley subtropical fruit-growing area in the south, which is dominated by extensive monocultures of macadamias, pecan nuts, avocados, bananas and pine and gum plantations (Figure 7). The detailed location of pellet collection sites within the study area are shown in Figure 8.

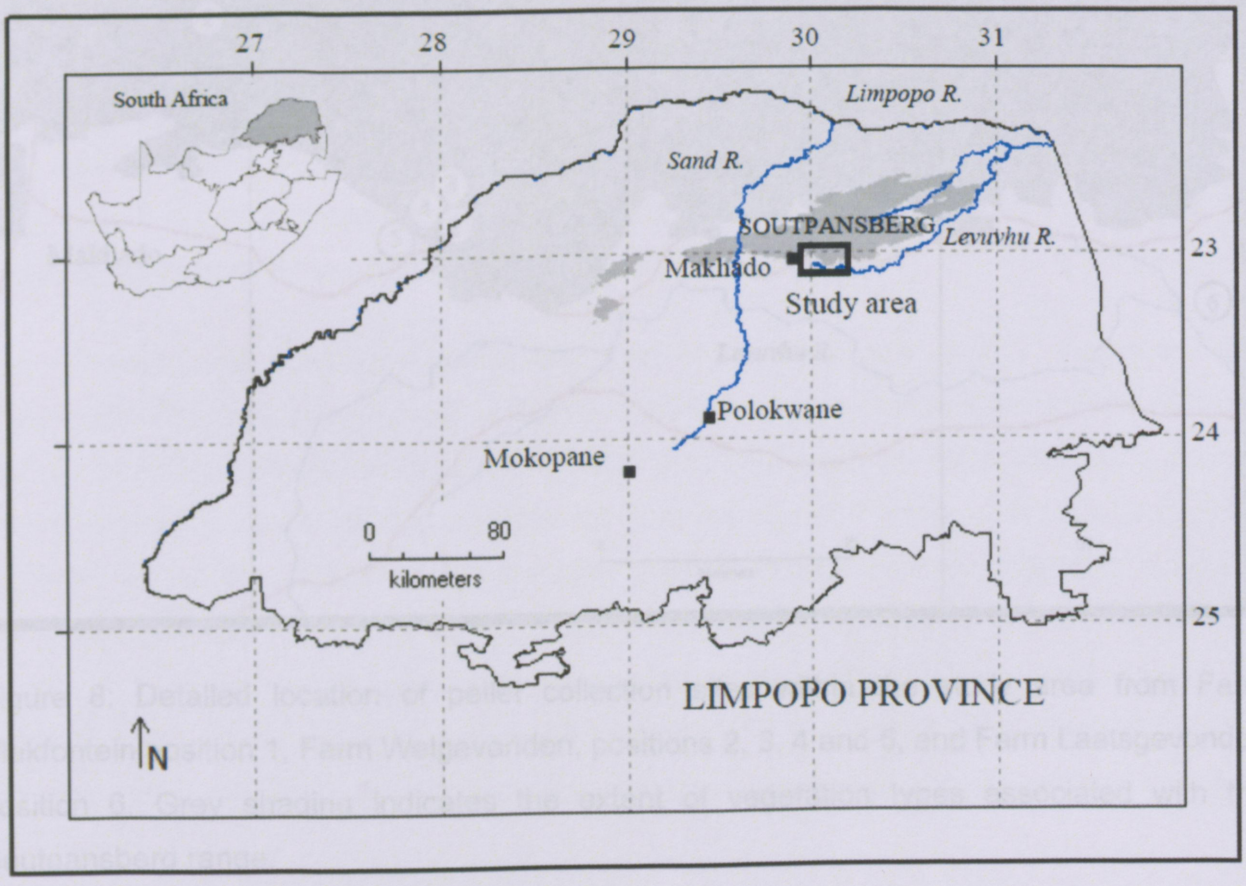


Figure 7: Map of study area showing location of study area in South Africa's Limpopo Province.

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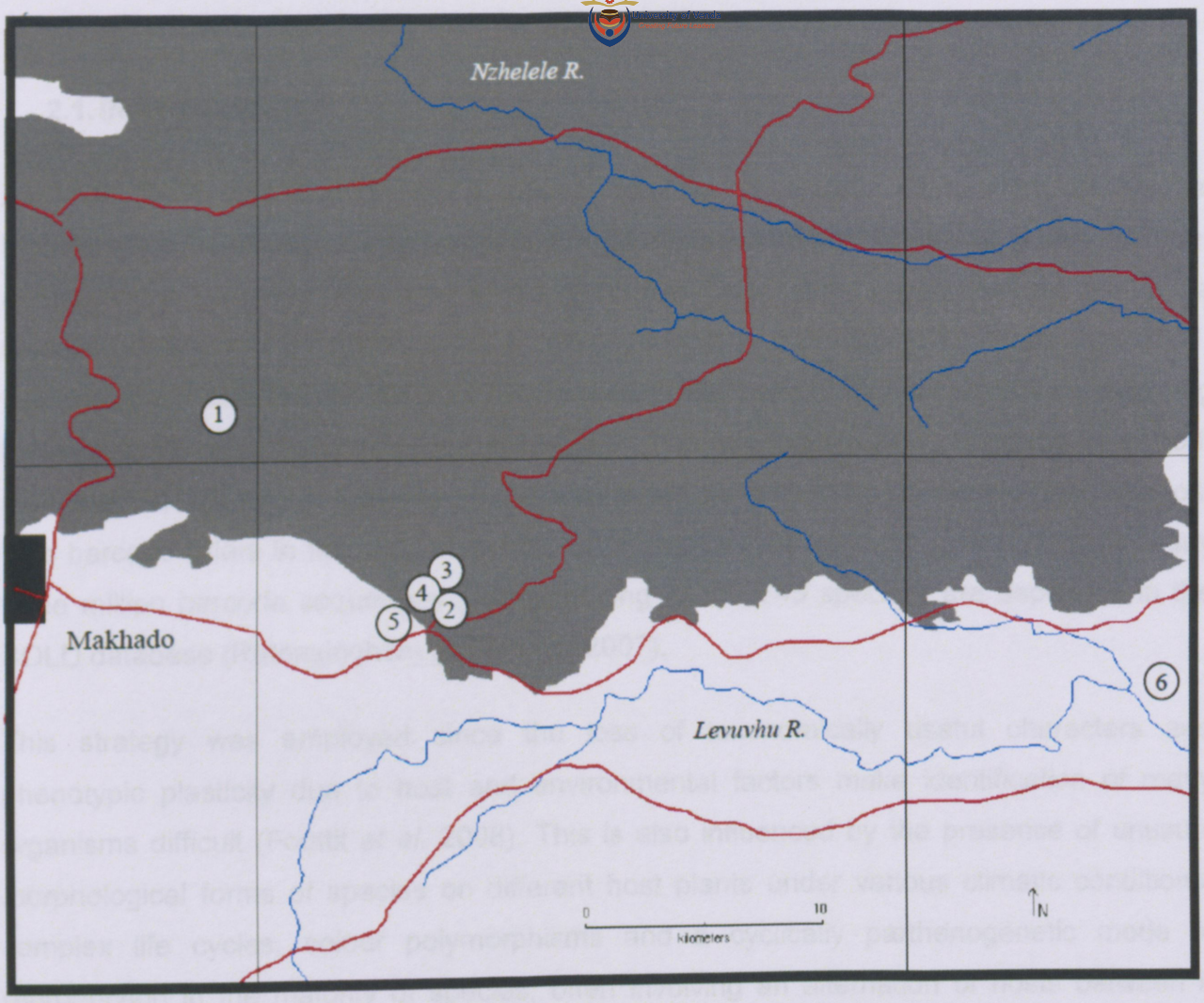


Figure 8: Detailed location of pellet collection sites within the study area from Farm Vlakfontein position 1, Farm Welgevonden, positions 2, 3, 4 and 5, and Farm Laatsgevonden position 6. Grey shading indicates the extent of vegetation types associated with the Soutpansberg range.

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2.1. INTRODUCTION

Although DNA sequencing has provided hope to accelerate exploration and global biodiversity assessments by sequencing a huge number of living organisms, a large number of organisms on earth still need to be described while other organisms are yet to be discovered (Baird and Hajibabaei 2012; Krishnamurthy and Francis 2012; Mora *et al.* 2011; Ratnasingham and Hebert 2007). DNA barcoding has been a successful tool for exploring biodiversity in the last years (Hebert *et al.* 2003). This method is based on the use of a short, standardized and highly variable genomic segment as a marker for species identification. This barcode refers to approximately 650 bp of mtDNA COI fragment and at present nearly three million barcode sequences (corresponding to 207 745 species) are deposited in the BOLD database (Ratnasingham and Hebert 2007).

This strategy was employed since the loss of taxonomically useful characters and phenotypic plasticity due to host and environmental factors make identification of many organisms difficult (Foottit *et al.* 2008). This is also influenced by the presence of unusual morphological forms of species on different host plants under various climatic conditions, complex life cycles, colour polymorphisms and a cyclically parthenogenetic mode of reproduction in the majority of species, often involving an alternation of hosts between a winter primary host, spring and summer secondary host(s) (Dixon 1998). It was evident that a new strategy be employed and Hebert *et al.* (2003) proposed the concept of DNA barcoding as a rapid and precise way of species discrimination of a broad range of biological specimens.

This chapter focuses mainly on the DNA barcoding of South African stinkbugs including those collected as part of this study in macadamia orchards in Limpopo Province. However the phylogeny to show how they are related to other insects on the BOLD systems from the same families has also been constructed and discussed in this chapter.

It is also known that there are genetic differences in morphologically similar species and genetic similarity of two morphological different species (Mayer and von Helversen 2001). Since there is lack of accuracy in animal identification using morphology, mtDNA COI has

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been established to serve as core of global bio-identification systems for animals (Hebert *et al.* 2003).

Universal primers (Folmer *et al.* 1994) which are used to target this region has also been used for phylogenetic analysis of the *Stegana ornatipes* species group (Diptera: Drosophilidae) (Lu *et al.* 2010), with morphological data to determine the phylogeny of Nereidiform polychaetes (Annelida) (Dahlgren *et al.* 2000), and in other projects (i.e Barrett and Hebert 2005; Hebert and Gregory 2005; Hubert *et al.* 2008; etc.)

In this study we focus on bio-identification of stinkbugs in South African Macadamia orchards, and also construct a phylogeny to see how they are related. In a research aimed at finding the relationship of family groups in Pentatomoidae based on morphology and DNA sequences, Grazia *et al.* (2008) used the 18S rRNA, 16S rRNA, 28S rRNA, and COI gene regions and found more information about the relationships between Pentatomoidae, however in their research they did not include any of stinkbugs used in this study.

2.2. RESEARCH QUESTIONS

- Is DNA barcoding an effective tool to identify both pest and non-pest species of stinkbugs occurring in macadamias (i.e. are between-species genetic distances significantly greater than within-species)?
- Can the barcode gene (COI) be used as a phylogenetic marker to determine the relationships between pest and non-pest species of stinkbugs occurring in macadamias?

2.3. METHODS

2.3.1. SAMPLE COLLECTIONS

Stinkbugs samples of 14 (Hemiptera) species (see Table 1) were collected from local macadamia plantations by Mr JN Steyn. Live insects were collected in any of the selected Macadamia Orchards. Some were preserved in 96-100% ethanol, while others were preserved by freezing. Before these insects were experimented with, they were sent to Dr M. Stiller who identified 12 of them to species level and two of them to genus level. They were then given to Ms K. Bohmann who took them to University of Coppenhagen, Denmark in to the Centre for Geogenetics, Natural History Museum of Denmark where they were anlysed.

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Table 1: List of Hemiptera species collected for this study

Specimen no	Species	Family, common name
1	*Nezara viridula*	Pentatomidae; green vegetable stinkbug*
2	*Bathycoelia natalicola*	Pentatomidae; two spotted stinkbug*
3	*Pseudotheraptus wayi*	Coreidae; coconut bug*
4	*Dalsira costalis*	Pentatomidae; stinkbug
5	*Solenostethium liligerum*	Scutelleridae; Thunberg
6	*Caura rufiventris*	Pentatomidae; stinkbug
7	*Coridius nubilis*	Dinidoridae; stinkbug
8	*Dysdercus nigrofasciatus*	Pyrrhocoridae; cotton stainer, a major pest of cotton
9	*Veterna* species	Pentatomidae; grass stinkbugs
10	*Anoplecnemis curvipes*	Coreidae; giant coreid bug
11	*Leptoglossus australis*	Coreidae; leaf-footed plant bug
12	*Bathycoelia rodhaini*	Pentatomidae; yellow-spotted stinkbug*
13	*Nezara pallidoconspersa*	Pentatomidae: Yellow-edged stinkbug*
14	*Solenostethium* species	Scutelleridae; Thunberg

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2.3.2. LABORATORY PROCEDURE

2.3.2.1. DNA EXTRACTION

The DNA was extracted using DNeasy Blood & Tissue Kit technique (QIAGEN). The protocol were done as follows: One or two legs (depending on the size) were cut into small pieces and placed in a 1.5 ml tube then 180 µl ATL buffer and 20 µl of proteinase K were added. The mixture was then mixed using the vortex mixer and incubated on a shaking water bath at 56ºC until legs are completely lysed. Following tissue digestion, 200 µl buffer AL were added to the sample, and were mixed by vortex for 15 seconds and before incubating at 70ºC for 10 minutes.

After incubation, 200 µl of ethanol (96-100%) were added to the sample and mixed thoroughly by vortex mixer. The solution was then be transferred into a DNeasy spin column fitted in 2 ml collection tubes and was centrifuged at 8000 rpm for a minute. The column containing the DNA was washed with 500 µl buffer AW1 and were centrifuged at 8000rpm for a minute; it was washed again with 500 µl buffer AW2 and centrifuged at 13000 rpm for 3 minutes new collection tubes were used in each step. The DNA was eluted in a new clean microcentrifuge tube by adding 50 µl Buffer AE directly on the centre of the column and spinning at 8000 rpm twice with position adjustments of the tubes in the microcentrifuge before storing at -18 ºC in a freezer.

2.3.2.2. POLYMERASE CHAIN REACTION (PCR)

The universal primers by Folmer *et al.* (1994) have been used. The forward primer is LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and the reverse primer is HCO2198: 5'-taaacttcagggtgaccaaaaaatca-3'. PCRs were performed in 25 µl reactions as indicated in Table 2. Using 1 µl of each primer at 10 mM, 2.5 µl of PCR buffer at 10 X, 2.5 µl of $MgCl_2$ at 25 nM, 0.2 µl of dNTP's mixture 25mM, 0.2 µl of Taq polymerase at 5 U/µl, with 1 µl of each sample with water to make 25 µl (see Table 2). For each seven reactions a PCR blank, consisting of all the reactans and water was used as a sample, was added (see Table 3).

Cycling were performed using a DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories) with the following cycle program: Initial denaturation at 95°C for 5 minutes followed by 50 cycles of 95°C for 15 seconds, 52°C for 30 seconds and 72°C for 30 seconds, final extension of 7 minutes at 72°C and 4°C forever.

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Table 2: Components of PCR reaction mixes

Volume used (µl)	DNA	10x Buffer	$MgCl_2$	DNTP 25mM	Taq	H_20	Primer F	Primer R
1 reaction	1	2.5	2.5	0.2	0.2	16.6	1	1
40 reactions	1	100	100	8	8	664	40	40

2.3.2.3. GEL ELECTROPHORESIS

PCR products were separated on 2 % agarose gel prepared as follows. For each 100 ml 1X TBE Buffer two grams of agarose powder were weighed and mixed. The mixture were heated until there is complete dissolution, this were achieved by turning the microwave to high. The mixture was cooled by washing the outside of bottle. Exactly 6 µl of Ethidium bromide10 mg/ml were added and mixed thoroughly. The mixture were transferred to the gel-casting tray with the comb already in position and allowed to solidify. The solidified gel was transferred to the running trays. The gel in the tray was covered with 1X TBE buffer. In the first well 3 µl of the 50 bp molecular weight marker were loaded and the samples were loaded from the second well onwards. The gel was run at 100 V, 400 mA and 150 W for 40 minutes. It was viewed under UV transilluminator and the picture taken was attached to the laboratory book.

2.3.3. SEQUENCING

All positive PCR products were sequenced at Macrogen (Seoul, South Korea) using the same PCR primers. All sequencing results were in the form of chromatographs.

2.3.4. ANALYSIS

For each taxon, multiple fragments obtained by sequencing with both forward and reverse primers; were edited and assembled using the Staden package (Staden *et al.* 2003). All assembled sequences were aligned in BioEdit v7.0.9 (Hall 1999). Sequences were run on BLAST algorithm (Altschul *et al.* 1990) to identify their closely similar sequences already deposited in GenBank via NCBI. The sequences which showed highest similarity to the groups outside the scope of this study were excluded.

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Additional sequences (in FASTA format) were downloaded from BOLD systems, and combined with assembled sequences to increase numbers in each of the families when constructing phylogenetic analyses. Phylogenies were inferred using the Neighbor-Joining method (Saitou and Nei 1987) using Kimura 2-parameter model (Kimura 1980) used with the assumptions that all species in this study are closely related, node reliabilities was assessed using bootstrap resampling analysis (1000 replicates). The Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980); and Maximum Parsimony analyses were also implemeted using the software MEGA 6 (Tamura *et al.* 2013)

The Evolutionary Divergence between and within Sequences and also between families were conducted using the Kimura 2-parameter model (Kimura 1980). Evolutionary analyses were conducted in MEGA 6 (Tamura *et al.* 2013).

2.4. RESULTS

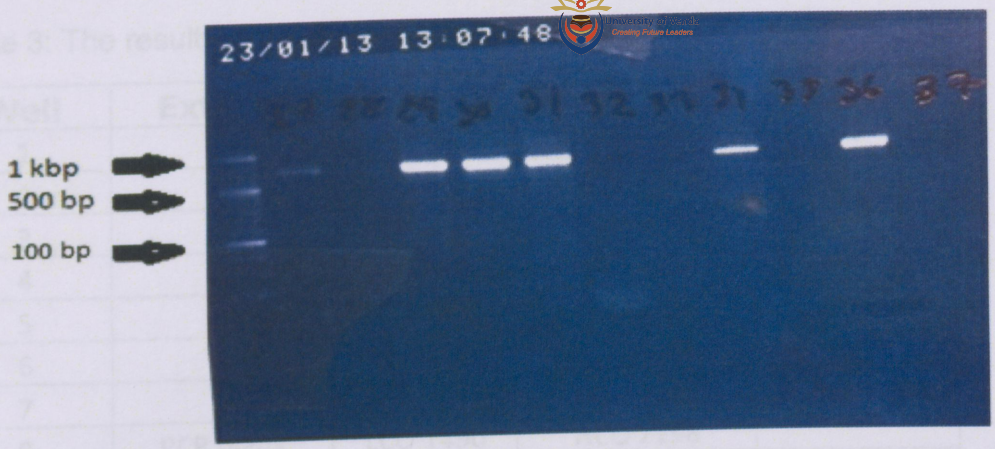
2.4.1. EXTRACTION AND AMPLIFICATION

PCR products separeated by gel electrophoresis are shown in Figures 9. a and b below. The failed samples were amplified again several times with no success and were then eliminated from this stage.



a)

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b)

Figure 9: Gel shots showing a) sample 1 to 26 and b) sample 27 to 37

The interpretations of the gel pictures above with the results are shown in Table 3 below. The key to the table + (positive sign) indicating the presence of a band which suggest that the reaction was successful whereas blank shows no band and that suggests a reaction failed.

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Table 3: The results of PCR of Insects after gel electrophoresis

Well	Extract	Primer F	Primer R	Pos/neg
1	1	LCO 1490	HCO 2198	+
2	2	LCO 1490	HCO 2198	+
3	3	LCO 1490	HCO 2198	+
4	4	LCO 1490	HCO 2198	+
5	5	LCO 1490	HCO 2198	
6	6	LCO 1490	HCO 2198	+
7	7	LCO 1490	HCO 2198	+
8	PCR blank	LCO 1490	HCO 2198	
9	8	LCO 1490	HCO 2198	+
10	9	LCO 1490	HCO 2198	
11	10	LCO 1490	HCO 2198	+
12	11	LCO 1490	HCO 2198	+
13	12	LCO 1490	HCO 2198	+
14	13	LCO 1490	HCO 2198	
15	14	LCO 1490	HCO 2198	+
16	PCR blank	LCO 1490	HCO 2198	
17	extra blank	LCO 1490	HCO 2198	
18	Pos. DNA	LCO 1490	HCO 2198	+
19	1	LCO 1490	HCO 2198	+
20	2	LCO 1490	HCO 2198	+
21	3	LCO 1490	HCO 2198	+
22	4	LCO 1490	HCO 2198	+
23	5	LCO 1490	HCO 2198	
24	PCR blank	LCO 1490	HCO 2198	
25	6	LCO 1490	HCO 2198	+ faint
26	7	LCO 1490	HCO 2198	+
27	8	LCO 1490	HCO 2198	+
28	9	LCO 1490	HCO 2198	+ faint
29	10	LCO 1490	HCO 2198	+
30	11	LCO 1490	HCO 2198	+
31	12	LCO 1490	HCO 2198	+
32	PCR blank	LCO 1490	HCO 2198	
33	13	LCO 1490	HCO 2198	
34	14	LCO 1490	HCO 2198	+
35	extra blank	LCO 1490	HCO 2198	
36	Positive DNA	LCO 1490	HCO 2198	+
37	PCR blank	LCO 1490	HCO 2198	

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Solenostethium liligerum and *N. pallidoconspersa* species did not amplify with the pair of primers used for this study, whereas both *Veterna sp.* and *B. natalicola* did amplify but both have failed to assemble in Staden package (Staden *et al.* 2003) and all these four species were left out of this project from here onwards.

The consensus sequences used for further analysis were imported using BioEdit v7.0.9 (Hall 1999) and all sequences are in Appendix 1.

BLAST algorithm (Altschul *et al.* 1990) was conducted for all the remaining species and the results are shown in Table 4. The table shows the reactions used for the selected samples and the family each sample falls under was shown in the next column, the best result from BLAST algorithm (Altschul *et al.* 1990) manually selected by the author were shown and its family was shown in the next column, the percentage similarity was also indicated in the table. Only *N. viridula* proved to be identifiable with 99% to already deposited sequences of the same species in GenBank; however the main reason why others cannot be identified with morphological same species is because few South African insects have been deposited in online databases.

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Table 4: Results of BLAST search showing the similarity between GenBank sequences with sample sequences from this project, Families of each species are shown on separate column.

Reaction	Samples	Family	Similarity	Species it is similar to	Family
1 &19	*Nezara viridula*	Pentatomidae	99%	*Nezara viridula* Isolate Neri-009	Pentatomidae
3 & 21	*Pseudotheraptus wayi*	Coreidae	85%	*Leptoglossus occidentalis*	Coreidae
4 &22	*Dalsira costalis*	Pentatomidae	87%	*Aelia americana*	Pentatomidae
6 &25	*Caura rufiventris*	Pentatomidae	87%	*Zicrona caerulea*	Pentatomidae
& 26	*Coridius nubilis*	Dinidoridae	85%	*Rhaphigaster nebulosa*	Pentatomidae
9& 27	*Dysdercus nigrofasciatus*	Pyrrocoridae	98%	*Rhynocoris segmentarius*	Reduviidae
11 & 29	*Anoplecnemis curvipes*	Coreidae	89%	*Anoplecnemis phasianus*	Coreidae
12 & 30	*Leptoglossus australis*	Coreidae	93%	*Leptoglossus gonagra*	Coreidae
13 &31	*Bathycoelia rodhani*	Pentatomidae	95%	*Bathycoelia indica*	Pentatomidae
15 & 34	*Solenostethium sp.*	Scutelleridae	86%	*Pachycoris torridus*	Scutelleridae

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2.4.3. PHYLOGENETIC RELATIONSHIPS

All trees shown below in Figures 10 to 12 were inferred in MEGA 6 (Tamura *et al.* 2013) and all samples from this study are indicated with a star (*), and the rest were individually downloaded from online database via www.boldsystems.org as Fasta files.

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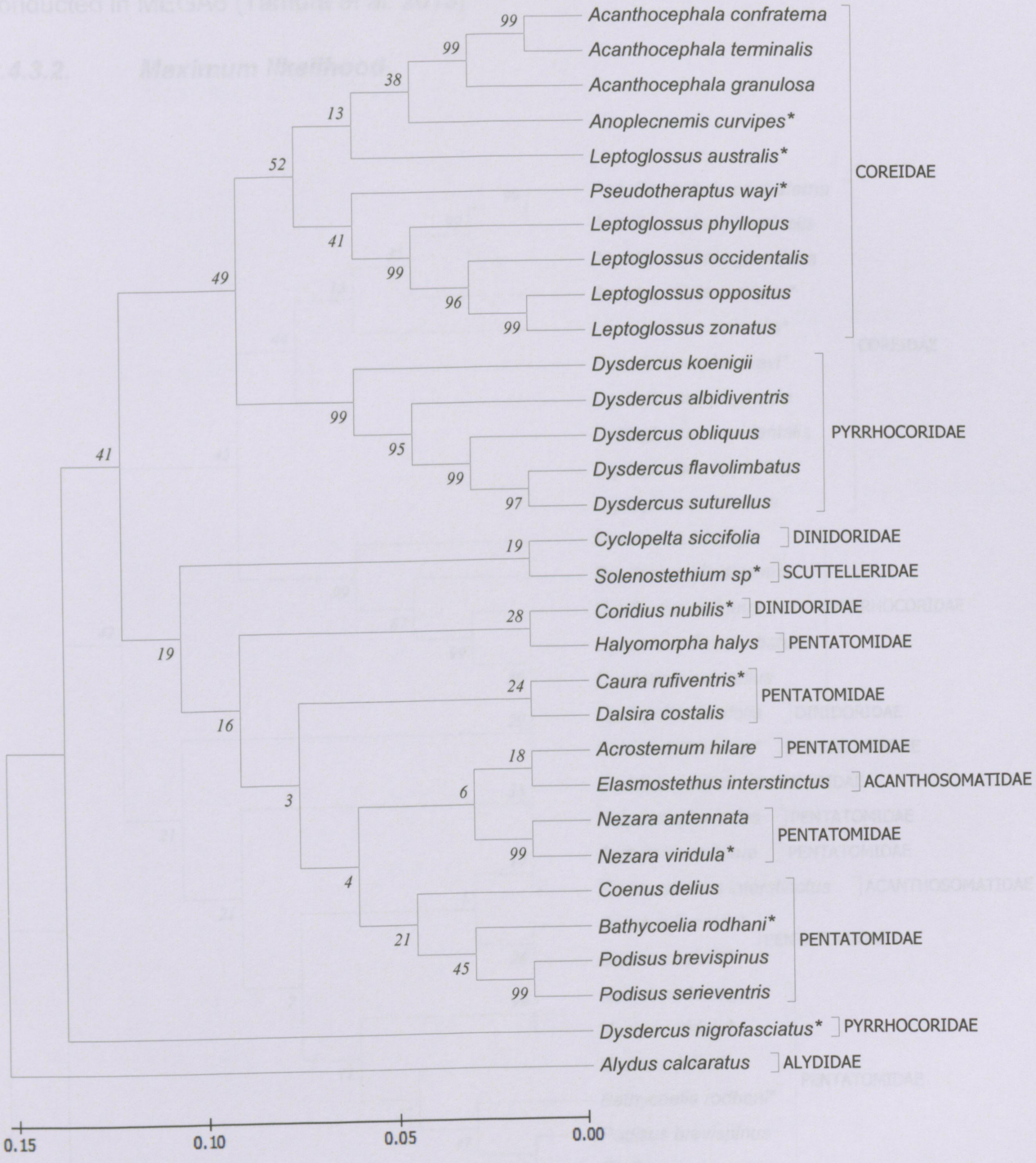


Figure 10: The evolutionary history of sequences was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1986). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions

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per site. There were a total of 658 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.* 2013)

2.4.3.2. *Maximum likelihood*

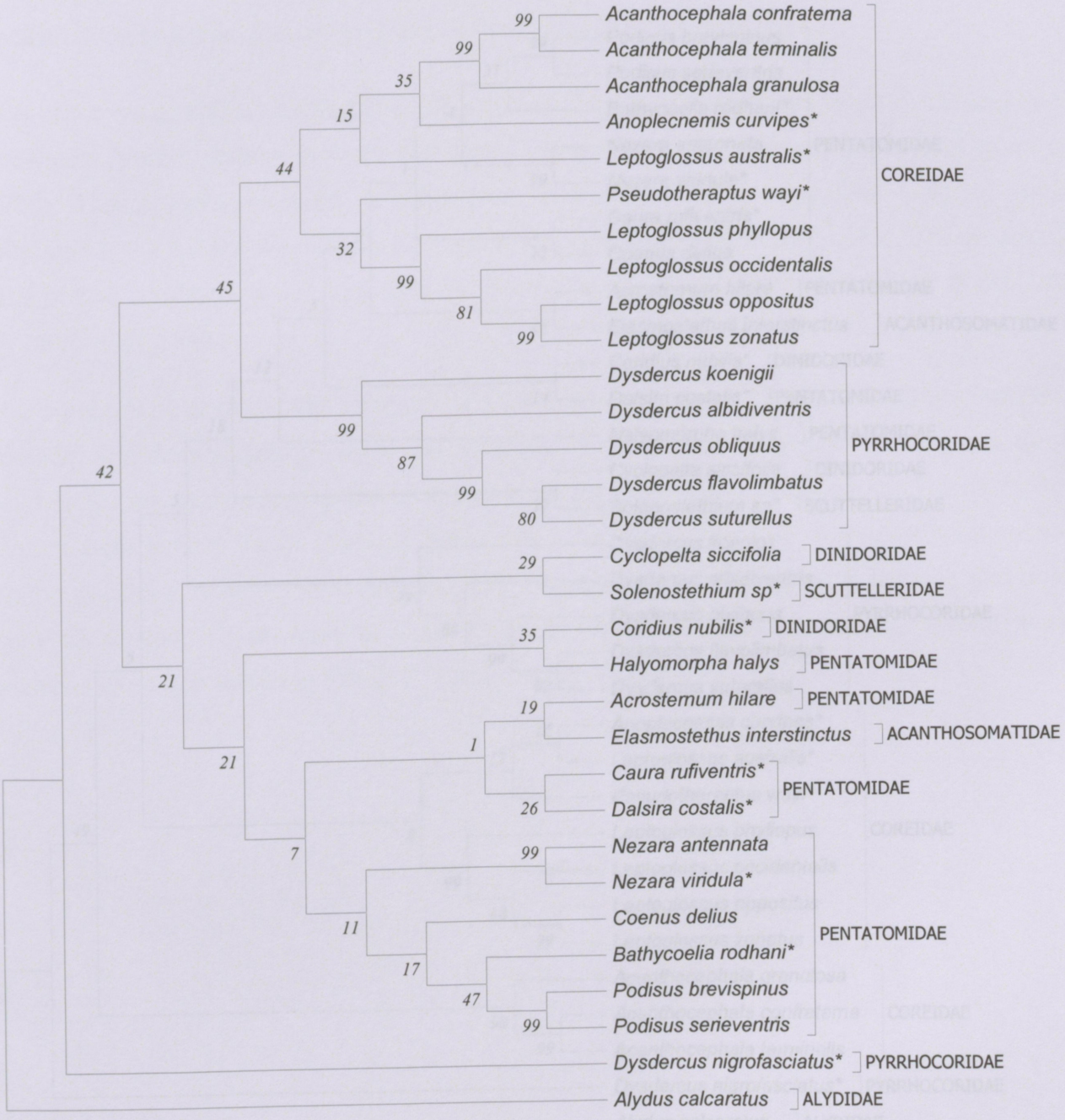


Figure 11: The evolutionary history of sequences was inferred using the Maximum Likelihood method, based on the Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood is shown. The percentages of replicate trees in which the associated taxa

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clustered together in the bootstrap test are shown next to the branches (Felsenstein 1985). Evolutionary analyses were conducted in MEGA 6 (Tamura *et al.* 2013)

2.4.3.3. Maximum Parsimony



Figure 12: The evolutionary history of sequences was inferred by using the Maximum Parsimony. The percentages of replicate trees in which the associated taxa clustered

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together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). Evolutionary analyses were conducted in MEGA 6 (Tamura *et al.* 2013)

2.4.4. DIVERGENCE MATRIX

The divergences within the groups and amongst the groups were calculated from the final dataset including downloaded sequences from www.Boldsystems.org.

The numbers of base substitutions per site from between sequences were calculated and shown in Table 5. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 658 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.* 2013).

The numbers of base substitutions per site from averaging over all sequence pairs between species and family groups were calculated and shown in Tables 6-7. Standard error estimate(s) are shown above the diagonal in Table 6. Analyses were conducted using the Kimura 2-parameter model (Kimura 1980). The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 658 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.* 2013).

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Table 5: Estimates of Evolutionary Divergence between Sequences. The divergence between samples is shown in the lower left triangle of the table and the diagonals indicate intraspecific distances based on two samples sequenced per species. All this was calculated in MEGA 6 (Tamura *et al.* 2013)

	Nezara viridula	*Pseudotheraptus wayi*	*Dalsira costalis*	*Caura rufiventris*	*Coridius nubilis*	*Dysdercus nigrofasciatus*	*Anoplecnemis curvipes*	*Leptoglossus australis*	*Bathycoelia rodhani*	*Solenostethium sp*
Nezara viridula [p]	0.000									
Pseudotheraptus wayi [c]	0.223	0.000								
Dalsira costalis [p]	0.175	0.222	0.000							
Caura rufiventris [p]	0.177	0.236	0.157	0.000						
Coridius nubilis [d]	0.188	0.235	0.188	0.192	0.000					
Dysdercus nigrofasciatus [r]	0.211	0.252	0.234	0.229	0.277	0.000				
Anoplecnemis curvipes [c]	0.231	0.211	0.242	0.252	0.227	0.256	0.000			
Leptoglossus australis [c]	0.223	0.203	0.204	0.236	0.224	0.249	0.209	0.000		
Bathycoelia rodhani [p]	0.146	0.226	0.166	0.182	0.203	0.231	0.210	0.220	0.000	
Solenostethium sp. [s]	0.237	0.240	0.225	0.224	0.227	0.264	0.243	0.221	0.250	0.000

Keys to the table above

P = Pentatomidae, C = Coreidae, D= Dinidoridae, R = Pyrrocoridae, and S = Scutelleridae

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Table 6: Estimates of evolutionary divergence sequence pairs between families. The genetic distances are shown in the lower left corner whereas their standard errors are shown on the upper right corner of the table.

	COREIDAE	PENTATOMIDAE	DINIDORIDAE	PYRRHOCORIDAE
COREIDAE		0.015	0.016	0.014
PENTATOMIDAE	0.230		0.014	0.013
DINIDORIDAE	0.247	0.209		0.016
PYRRHOCORIDAE	0.210	0.204	0.229	

Table 7: Estimates of Average Evolutionary Divergence over Sequence Pairs within families and their standard errors are shown.

FAMILIES	DIVERGENCE	STANDARD ERROR
COREIDAE	0.193	0.013
PENTATOMIDAE	0.173	0.011
DINIDORIDAE	0.256	0.023
PYRRHOCORIDAE	0.121	0.009

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2.5. DISCUSSION

2.5.1. DNA BARCODING

The importance of BLAST algorithm (Altschul *et al.* 1990) is very important in helping identification of organisms to their specific genera with respect to similar available sequences in online databases. From BLAST algorithm (Altschul *et al.* 1990) it must be understood that more than 98% similarity obtained matches the sample to the correct the species (i.e. *N. viridula*), more than 90% similarity obtained matches the sample to the correct the correct genus (i.e. Bathycoelia and Leptoglossus), more than 80% similarity obtained matches the sample to the correct the correct Family level (i.e. *P. wayi, Anoplecnemis curvipes, Dalsira costalis, Caura rufiventris,* and *Solenostethium sp.*).

However similarity abtained which is lower than 90 % indicates that the relevant sequences are not available on online databases. However *Coridius nubilis* (Hemiptera: Dinidoridae) shows 85% similarity to *Rhaphigaster nebulosa* (Hemiptera: Pentatomidae) in a different group calling for for need of molecular study in South African Dinidoridae species, these is also influenced by that no organism from this famly can be found on online databases.

However in the case of *Dysdercus nigrofasciatus* which shows 98% match with *Rhynocoris segmentarius* which is a very high similarity, the fact that difference in standard barcoding sequence between species is a bit more than 2 % then it is highly likely this is probably one species. Based on the understanding of the geographical location of *Rhynocoris segmentarius* indicated on BOLD (Ratnasingham and Hebert 2007) which is not in Africa and the absence of *Dysdercus nigrofasciatus* record in any database, it can be strongly indicated that the morphological identification provided for this species is incorrect and this underlines an important benefit of barcoding, i.e. it allows correction of misidentifications based on morphological identifications which are often difficult. This suggest that there is a gap in identification of this insects both using the molecular and morphological methods.

The intraspecific distances (discussed below) in Table 5 support becomes the strength of reference in suggesting that morphological classification of this two insects need to be revised. Weirauch *et al.* (2014) gave a full morphological identification of family Reduviidae and in no way discussed how the insects in this family vary in relation to insects from family Pyrrhocoridae; as there is little description of *Dysdercus nigrofasciatus* by Schaefer and

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Pannizzi (2000) it is understandable that the morphology of the two insects might suggest varios possibilities in identifications.

The families Reduviidae and Pyrrhocoridae are both from superfamily Pyrrhocoroidea (Lis *et al.* 2011) whereas Dinidoridae and Pentatomidae in the same superfamily Pentatomoidea. The close relationship between the families suggest the similarity exist between our samples which might be enough to confuse identifications morphologically, also there is a relationship of all this insects in this study since they are from suborder Pentatomorpha (Order Heteroptera) the second largest suborder (Li *et al.* 2005). This provides enough evidence for revision of this group of insects based on both morphology and molecular methods.

The DNA barcoding is based on the standard short COI sequence and relies on the idea that genetic variation within species (intraspecific distances) is smaller than genetic variations between species (interspecific distances) (Hajibabaei *et al.* 2007). The genetic distances as shown, in Table 4 (taken from two samples), shows that DNA Barcoding can work successfully since all the samples within the species aligned perfectly with no base difference when consensus sequences were created, giving a zero intraspecific distances. However the sample size is too small to make a conclusion based on it. Comparing the genetic variations between species and to the variations of the families and using the standard errors the will always be a gap this gap is termed barcoding gap (Meyer and Paulay 2005) this, since there is no overlapping of the interspecific and intraspecific divergence, supports the success of Barcoding.

However the finding by Meier *et al.* (2006) implies to us that in order to have success in DNA Barcodes is by finding more barcodes of many individuals of the same species. Since our study was only to find Barcodes to use them as reference sequences for diet analysis of bats, which its success will be discussed in details in the next chapter, the study has achieved a primary goal as Hajibabaei *et al.* (2007) agreed that DNA Barcodes can be a useful tool in taxonomy as used for routine identification of specimens and comprehensive taxonomic investigations. It is this study which will open eyes to the South African molecular taxonomists to try and identify these pest insects using Barcodes.

However it is important to mention that DNA barcodes has been determined as a useful tool in identification of Hemiptera and thought to advance in pest management, regulatory and environmental applications and also reveal species that require further taxonomic research

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(Park *et al.* 2011). Tembe *et al.* (2014) has also shown that the efficiency of the technique has been confirmed in infraorder Pentatomomorpha (Hemiptera: Heteroptera); this includes the work by Jung *et al.* (2011) and Park *et al.* (2011).

From this study it is important to point out that there is a need of the South African Hemiptera insects to be identified using molecular techniques and that the sequences obtained should be deposited in online databases so they can be used in species identifications and references for future reseachers.

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2.5.2. DIVERGENCE

Hajibabaei *et al.* (2007) indicated that DNA can be used in population genetics investigations where barcodes will provide first signal of the depth and type of population divergences. In their study they also emphasized that DNA Barcoding can be used in many population diversity experiments in several species.

In this study barcodes were therefore also applied for divergences; the divergence within families has been supported by individuals from this study amongst themselves. The representatives of Pentatomidae have a maximum genetic divergence (between *N. viridula* and *Caura rufiventris*) which is inside within the standard error of Pentatomidae divergence within species, and the same applies to Coreidae.

There cannot be a comparison on Pyrrocoridae since only one member of that family was part of this study. The standard error for Dinidoridae family is highest at 2.3 %; this might be very high because *Coridius nubilis* from our study is included as a member of the family whereas there is 85 % chance that it belongs to family Pentatomidae. More samples of this species need to be collected before a complete reclassification from Dinidoridae. In the content of this study family Scutelleridae was never entertained in any way this might be because *Solenostethium liligerum* and other *Solonostethium species* were originally classified in family Pentatomidae (based on www.boldsystems.org) until after performing BLAST algorithm (Altschul *et al.* 1990) and downloading articles with species of this genus which shows that in fact *Solonostethium* species belong to Family Scutelleridae.

There has been no study related to divergence of heteropterans hence this study can be used a basis of divergence for these bugs (Hemiptera: Heteroptera: Pentatomidae). In South Africa both morphology and molecular studies of this insect, which includes major pests

of agriculture of macadamia, guava, avocado and other industries, have not been studied with reference to divergence between and within families of Order Hemiptera. Schoeman (2009 and 2013) indicated that an IPM of these insects is needed especially in Macadamia and avocado industries; hence their relationships are very important to be known.

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2.5.3. PHYLOGENETICS

Lis *et al.* (2011) has examined the comparative studies on pentatomomorphan bugs which are in the infraorder in which all samples for this study falls under. However their study was to examine nucleotide divergence along with nucleotide composition and substitution patterns of this infraorder based on the 12S and the 16S mitochondrial rDNA fragments. Though they could have used the study for phylogenetics, they were focusing on how useful a museum specimen can be in molecular analyses.

Hajibabaei *et al.* (2007) explained that DNA Barcodes cannot be used for phylogenetics but can however be used to complement it by providing background information that will be helpful in the selection of taxa for further analyses. However in this study Phylogenetics based on thee COI regeion was carried out using three methods in Mega 6 (Tamura *et al.* 2013); the methods used are Neighbor Joining, Maximum Likelihood and Maximum Parsimony. It is important to first mention the fact that the species from online databses which matched with samples from this study are not part of the phylogenies constructed since most were shorter than 658bp which is the size of our alignment (except *N. virula* and *Rhynocoris segmetarius* excluded by being considered the same), the alignment has useful information at both ends which prevented it to be trimmed since information could be lost. Only *Leptoglossus occidentalis* was useful in both instances.

The main reason of doing phylogenetics was to test monophyly of two important pest families which are Pentatomidae and Coreidae. They didn't produce a true monophyletic tree when tested when sequences are combined in the final dataset there is paraphyly and polyphyly in both Pentatomidae and Coreidae families.

In neighbour joining tree the Coreidae insects have produced a formation of a clade with a 52 percent bootstrap support which is not that good but at least above 50 percent. The further break down of this group in two sees *Leptoglossus australis* being separated from the organisms which shares the genus with it, those organisms are seen to form a well supported clade.

2.6. CONCLUSIONS

Sequence data for the COI gene did allow the correct identification of the hemipteran pest *N. viridula* making DNA barcoding a useful tool for rapid identification of this major group of

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pests of the macadamia industry, this conclusion may be after the intraspecific divergence was so low as compared to interspecific divergences.

However the whole idea can be overshadowed by the fact that almost all South African Hemiptera are not listed in GenBank changing the focus from identifications using molecular techniques to DNA barcoding of insects identified by hemipteran specialists.

Sequence data for the COI gene from hemipteran species and genera did not provide a useful phylogenetic marker to allow an improved phylogenetic analysis of Southern African bugs (Hemiptera) this is partially because the Barcodes failed to confirm the monophyly of the two stinkbug families, Pentatomidae and Coreidae (besides the fact that Coreidae did form monophyly in two of the three methods used) and that the bootstrap supports are relatively very low and most of the clades cannot be confirmed.

Sequence data for the COI gene from hemipteran species collected in macadamia orchards and downloaded from online databases proved to be a useful tool to initiate the genetic distances within and between the hemipteran families. This is because only few individuals were used in the study and this therefore suggest further analysis using a bigger pool of samples

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3.1. INTRODUCTION

More than US$ 1.2 million (~R13.9 million) worth of rice is protected annually in Thailand by bats (Wanger *et al.* 2014) with their roles as pest predators. In South Africa, ongoing research is exploring the question as to whether they can reduce the R50 million losses in macadamia and avocado orchards from pest stinkbugs which were estimated in 2013 (Schoeman 2013). The first step is to provide definitive proof that pest stinkbugs are an important component of the diet of bats foraginig in macadamia orchards. This chapter then focuses on this initial question concerning the role of bats as biological control of pests as a way forward of implementation of IPM in macadamia orchards in South Africa.

3.1.1. BIOLOGICAL CONTROL VERSUS CHEMICAL CONTROL

In an argument of the classical biological control the factors such as i) the irreversibility of alien introductions, ii) the possibility of host switching to innocuous native or beneficial species, iii) dispersal of biological control agents to new habitats, and iv) the lack of research on the outputs of the biological control agents, Howarth (1983) rose an important point not to employ biological control agents started to impact (Follett *et al.* 2000).

Then an issue of the non-target effects caused by introductions of the biological control agents prompted Follett *et al.* (2000) to re-examine Pandora's box, and they found out that non-target effects include extinction of native moths, disappearance of native predators of the target pests and attack of non-target and non-pest species, however they found out that the data collected was a little biased since the investigators did not know the magnitude of non-target population suppression and in some instances the reports were based on casual observations and circumstantial evidence.

However in the research to fight destructive pest insects, Staments, (in Snyder and Miles 2004) found out that many chemicals used for control of these pests harm the non-targeted organisms, pollute water, and impair human health. From that the employment of biological control was recommended and hence they suggested *Metarhium anisopliae,* a biological control agent of termites and ants, after they found out it could attract and kill them (Snyder and Miles 2004).

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The use of pesticides and planting of genetically modified crops affects the huge numbers of natural enemies of agricultural pests, which can be used as biological control agents of these pests (McCracken *et al.* 2012).

The exotic ant *Pheidole megacephala* has been recorded as a predator of the eggs of *N. viridula*, but its impact on the eggs was not enough for the ant to be considered as a biological control agent of this pest (Seymour and Sands 1993). The parasitoid, *Trichopoda giacomellii* (Diptera: Tachinidae) which was first used in Argentina (La Porta 1987), was also tested as a biological control agent of *N. viridula* (Sands and Coombs 1999) and had been effective when it was with *Trissolcus basalis* in Australia and (Coombs 2002).

Both biological control and chemical control of stinkbugs has been attempted in South Africa. The biological control of *N. viridula* and possibly some of the other Pentatomidae in South Africa was established by introducing the egg parasitoid *Trissolcus basalis* and *Trichopoda pennipes* (Van den Berg 1995), and however there is no success recorded up to now.

Riechert and Lockley (1984) thought that generalist predators such as spiders can limit exponential increases in pest populations and need to form a part of integrated pest management strategies in agro-ecosystems, and insectivorous bats are concluded generalist predators (Kunz *et al.* 2011). However Dippenaar-Schoeman *et al.* (2001b) in their surveys suggested that more data on the feeding behaviour and community structures of spiders are needed to conclude if they can be used in the pest control programs of insects in macadamia orchards in South Africa.

Since bats are generalist predators and major predators of night-flying insects including crop pests and have been recorded to feed on stinkbugs (Jones *et al.* 2009), it is believed that they can be used as biological control agents in macadamia orchards. Bats have been recorded to feed on stinkbugs even in the southern parts of Africa (Bohmann *et al.* 2011; Taylor *et al.* 2013).

Insectivorous bats are evaluated if they can be attracted to macadamia orchards and control stinkbugs in this chapter. To achieve that, the DNA of stinkbugs in South African macadamia orchards was analysed (in the previous chapter) as to use as references to find out which species of insectivorous bats in South Africa feed on stinkbugs. The study was based on the diet analyses of bats for stinkbugs in they feed on to determine if they be used as biological control agents of stinkbugs in macadamia orchards in South Africa.

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The study of diets of insectivorous bats dates back to 1929, when Poulton (1929) studied the diets of British insectivorous bats, but then the investigation of the diet was achieved by collecting the guts of the bats.

3.1.2.1. Faecal dissection analysis

The standard faecal analysis methods involves soaking dried pellets in ethanol in a petri dish and teasing them apart under a dissecting microscope at a magnification of 30 X., then visually determing the percentage of arthropods within the pellet (Murray and Kurta 2002; Whitaker and Kunz 1988; Whitaker 2004).

The method has been practiced by Rodríguez-Durán and Lewis (1987) who analysed faecal pellets from Puerto Rico, and Silva-Taboada (1979), who examined the contents of stomachs from Cuba, but it has however produced results with many differences to the results to that of Rolfe (2011), who also analysed diet of bats in Puerto Rico. However these methods are not perfect as other insects are soft bodied and can be digested completely.

3.1.2.2. Molecular methods

For insectivorous bats, which typically eat a great diversity of prey and which chew and digest their prey thoroughly, DNA-based approaches to diet analysis may provide the only means of assessing the range and diversity of prey within faeces (Zeale 2010). Through PCR amplification of DNA sequences unique to prey species, identifications can be achieved even within highly degraded samples such as those found in faeces, gut contents, or regurgitates (King *et al.* 2008). Very short fragments between 100-250 bp of the complete COI barcode region can be efficient for species-level resolution and can be easily recovered from degraded samples.

McCracken *et al.* (2012) documented the significant association between numbers of Brazilian free-tailed bats (*Tadarida brasiliensis*) consuming corn earthworm (CEW) moths (*Helicoverpa zea*) and seasonal fluctuations in CEW populations using a real time PCR faecal DNA assay.

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In his Ph.D research Zeale (2010) recovered prey DNA from all faecal samples and subsequent cloned and sequenced the PCR products, followed by a comparison of sequences to a reference database. Bohmann *et al.* (2011) demonstrated the use of a powerful alternate tool, the use of the Roche FLX sequencing platform to deep-sequence uniquely 5′ tagged insect-generic barcode cytochrome c oxidase I (COI) fragments, that were PCR amplified from faecal pellets of two free-tailed bat species *C. pumilus* and *M. condylurus* (Family: Molossidae).

3.2. RESEARCH QUESTIONS

1. What do bats in South African macadamia orchards feed on?
2. How does the diet of six species of bats from a macadamia agro-ecosystem compare with published literature for these species and does it include pest species of macadamia including pest stinkbugs of the Pentatomidae and Coreidae complex and pest moths of the Torticidae complex?
3. Do different species of bats having different foraging strategies feed on different pest insects having different flight behaviour?
4. What are the pro's and con's of NGS versus conventional microscopic dissection of fecal pellets in determining the diet of bats?

3.3. METHODS

3.3.1. SAMPLE COLLECTIONS

All samples were collected in macadamia orchards and areas forming the borders of macadamia orchards. In all cases where faecal pellets were obtained from day or night roosts (for three species), they were collected onto 1 x 2 m boards covered in Clingfilm placed under the position of roosting bats approximately one week prior to collection. Pellets of the remaining four species were obtained from captured individual bats that were held individually in cloth bags until they yielded sufficient numbers of pellets.

Aerial bats were captured by the use of the harp trap and their pellets were collected before the bats were released. Bats which roost in houses were not captured but fresh and clean boxes were placed below the roosts and fresh pellets were collected the next morning. The pellets were preserved by refrigerating. The species of bats which the sample was collected from were recorded as well as the dates and method used to gain access to the pellet.

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3.3.2.1. DNA EXTRACTION: QIAAMP DNA STOOL KIT

One dropping from storage was taken and the excess ethanol was swabbed with a contamination free paper towel; it was weighed and the weight was recorded. The droppings were placed in a 2ml centrifuge tube on ice. The oven was turned on at 70 °C. All big pellets were cut into halves; this is because the protocol is optimized for a pellet between 0.002 g and 0.005 g. Then 1.4 ml (2 x 700 µl) of Buffer ASL was added to the stool sample. It was mixed by vortex for 1-3 minutes continuously or until the stool sample is thoroughly homogenized.

The suspension was heated for 10 minutes at 70°C in a hot block, before it was mixed by vortexing for 30 seconds. It was centrifuged at 13,000 rpm for 1 minute. Exactly 1.2 ml (2 x 600 µl) of the supernatant was pipetted into a new 2 ml centrifuge tube. Half of an Inhibit EX tablet was added to the sample and was mixed by vortexing immediately and continuously for 1 minute or until the tablet was completely suspended. The suspension was incubated for 1 minute at room temperature to allow inhibitors to absorb to the Inhibit EX matrix. The mixture was centrifuged at 13,000 rpm for 3 minutes to pellet inhibitors bound to Inhibit EX.

The supernatant was transferred into a new 1.5 ml centrifuge tube and the pellet was discarded. The transferred mixture was centrifuged at full speed for 3 minutes. Twenty microliters of Proteinase K was added into a new 1.5 ml centrifuge tube, before adding 400 µl of supernatant from the previous step. Then about 400 µl of Buffer AL was before mixing by vortexing for 15 sec, and this mixture were incubated at 70°C for 15 minutes in a hot-block. The mixture was centrifuged briefly to remove drops from the inside of the tube lid. Exactly 400 µl of ethanol (96-100%) were added to the lysate and were mixed by vortex. To remove any condensation on the lid of the centrifuge tube the mixture was centrifuged briefly.

The QIAamp mini spin column was placed in a new 2 ml collection tube and labelled. Only 600 µl of the lysate was applied carefully to a QIAamp spin column without moistening the rim. The column was centrifuged at 13,000 rpm for 1 minute. Centrifugation is set to be achieved only until the QIAamp spin column is empty. The spin column was placed in a new collection tube while discarding the collection tube containing the filtrate. This step was repeated with the remaining lysate using the same QIAamp mini spin column.

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Care was taken when adding 500 µl Buffer AW1 and the column was centrifuged at 13,000 rpm for 1 minute. A spin column was placed in a new 2 ml collection tube and the collection tube containing the filtrate was discarded. Care was taken again when adding 500 µl Buffer AW2 and the column was centrifuged at 13,000 rpm for 3 minutes. The filtrate in the collection tube was discarded and the collection tube was reused. The column was placed back in the collection tube and centrifuged at 13,000 rpm for 1 minute. The spin column was placed in a new 2 ml collection tube (or microcentrifuge tube) and the old collection tube was discarded and 50 µl of Buffer AE pipetted directly onto the spin column membrane. The cap were closed and incubated for 5 minutes at preferably 40 degrees on hot block then centrifuge at 13,000 rpm for 1 minute to elute DNA. The DNA eluted was transferred new labelled 1.5 ml tube and stored at -20 ^{0}C.

3.3.2.2. Polymerase Chain Reaction (PCR)

As for stinkbugs except that insect tagged primers by Zeale (2010), MZARTF+R for short fragment of COI were used, all PCRs were performed in 25 µl and the following cycling conditions were performed using a DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories): Initial denaturation at 95°C for 5 minutes followed by 50 cycles of 95°C for 30 seconds, 52°C for 30 seconds and 72°C for 30 seconds, followed by a final extension of 7 minutes at 72°C and 4°C forever. However for each reaction a different primer pair was used. Each sample was processed until it gives at least two positive PCR products with unique primer pairs. Five microliters of the PCR products were separeted in 2 % Agarase gel.

3.3.2.3. LIBRARY BUILD PREPARATION

3.3.2.3.1. PCR POOL

The pool was prepared based on the band visibility after running the gel. For all solid and brighter bands only one microliter were used; and for solid and bright band five microliters were used while the others with faint and slurry bands 15 microliter were added. All samples with at least two positive PCR products were pooled into a 15 ml tube. Only two PCR products were pooled per sample, and the samples with less than two PCR products were not considered for any further analysis. Appendix 2 indicates the exact volumes used for the preparation of the pool.

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The pool was purified in a 15 ml tube, the volume of the pool was determined and 5 times volume PB buffer was added and mixed by vortexing. The mixture was centrifuged at 10000 xg for 1 minute; this step was achieved by centrifuging 600 µl per time and emptying the collection tube. The spin column were transferred to new collection tube and 700 µl PE buffer were added and centrifuged 13000 x g for 1 minute and discard the collection tube.

The empty spin column was centrifuged at maximum speed for 2 minutes to dry. The spin columns were transferred into a new microcentrifuge tube and the lids were cut off before adding 50 µl of heated buffer EB (37 ^{0}C) and incubating at 37 ^{0}C for 5 minutes and centrifuging at maximum speed for 1 minute to collect DNA. The tube was turned and the centrifuging was repeated. Before running the pool in the bioanalyzer its concentration was measured using a Qubit® 2.0 Fluorometer following the manufacturer's manual.

3.3.2.3.2. BIOANALYZER

The purified PCR pool was run on the Agilent 2100 Bioanalyzer in order to see how much total DNA (227 bp fragments compared to primer-dimers) was present after pool purification. The Bioanalyzer looked so good that was no need to cut out the 227 bp fragments on the XT (see Figure 14), and the next step started straight away was library building procedure.

3.3.2.3.3. LIBRARY BLUNTING PROCEDURE

In a PCR tube 30 µl QIAquick purified PCR pool was mixed with 12.5 µl ddH_2O, then 5µl NEBNext 10x end repair buffer, and lastly 2.5µl NEBNext end repair enzyme mix were added to give a total of 50 µl(s) reaction. In the different PCR tube the same volumes of reagents were added with pool replaced by water as a blank control. All the tubes were centrifuged quickly and incubated on a thermocycler at 12°C for 20 minutes and at 37°C for 15 minutes before holding at 4°C.

The library was purified in a 2 ml microcentrifuge tube. The library and library blank were transferred to different microcentrifuge tubes and 250 µl of PB buffer were added to both tubes and mixed by a vortex. The mixtures were centrifuged at 10000 xg for 1 minute. The spin column was transferred to new a collection tube and 700 µl PE buffer were added and centrifuged 13000 xg for 1 minute and the collection tube was discarded. The spin column was centrifuged at maximum speed for 2 minutes to dry.

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The spin column was transferred into a new microcentrifuge tube and the lid was cut off before adding 50 µl of heated buffer EB to 37 ^{0}C and incubating at 37 ^{0}C for 5 minutes and centrifuging at max speed for 1 minute to collect DNA. The tube was turned and the centrifuging was repeated.

3.3.2.3.4. LIGATION

In a PCR tube 30 µl of Libraries (library and the blank) were mixed with 10ul quick ligation 5x buffer, then 5µl DNA adaptors, and lastly 5µl Quick T4 ligase to give a total of 50 µl(s). All the tubes were centrifuged quickly and incubated on a thermocycler at 20°C for 20 minutes before holding at 4°C. The ligated library was purified in a 2 ml microcentrifuge tube. The library and blank were transferred into different microcentrifuge tubes and 250 µl PB buffer were added to both tubes and mixed by a vortex. The mixture was centrifuged at 10000 x g for 1 minute; this step was achieved by centrifuging 600 µl per time and emptying the collection tube. The spin columns were transferred to new collection tube and 700 µl PE buffer were added and centrifuged 13000 x g for 1 minute and discard the collection tube. The empty spin column was centrifuged at maximum speed for 2 minutes to dry. The spin columns were transferred into a new microcentrifuge tube and the lids were cut off before adding 42 µl of heated buffer EB (37 ^{0}C) and incubating at 37 ^{0}C for 5 minutes and centrifuging at max speed for 1 minute to collect DNA. The tube was turned and the centrifuging was repeated.

3.3.2.3.5. FILL IN

In a PCR tube 42 µl of purified Library were mixed with 5 µl Adapter fill-in reaction buffer and 3 µl Bst DNA polymerase were added to give a total of 50 µl(s) reaction. The same were done with the blank. All the tubes were centrifuged quickly and incubated on a thermocycler at 65°C for 20 minutes and at 80°C for 20 minutes. The tubes were frozen overnight to stop the fill in reaction.

3.3.2.3.6. PCR

An index PCR was prepared with 5 µl of the fill in products by adding 5 µl of 10 X Taq Gold buffer, 5 µl of 25mM $MgCl_2$, 0.5 µl of 25mM dNTPs, 2.5 µl of BSA, 0.4 µl of Taq gold (enzyme) 5 u/µl, 1 µl of InPE (F) (Index Primer Paired End), 1 µl of Index R (1-8) and 29.6 µl of ddH_2O to five tubes each with 5 µl of the fill in products to run a 50 µl reaction.

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The cycling conditions were performed using DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories): Initial denaturation at 95°C for 5 minutes followed by 10 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes and 4°C until stopped. The index PCR was prepared with library blank in the other tube but only one replicate was done for the Library blank. The negative control for this reaction was done by replacing the fill in products with water.

The amplified library was purified in a 2 ml tube. The volume of the amplified library was determined and 5x volume PB buffer was added and mixed by a vortex. The mixture was centrifuged at 10000 x g for 1 minute; this step was achieved by centrifuging 600 µl per time and emptying the collection tube. The spin column were transferred to new collection tube and 700 µl PE buffer were added and centrifuged 13000 x g for 1 minute, and the collection tube discarded.

The empty spin column was centrifuged at maximum speed for 2 minutes to dry. The spin columns were transferred into new microcentrifuge tubes and their lids were cut off before adding 35 µl of heated buffer EB (37 ^{0}C) and incubating at 37 ^{0}C for 5 minutes and centrifuging at max speed for 1 minute to collect DNA. The tube was turned and the centrifuging was repeated.

3.3.2.3.7. LIBRARY FINALISATION

The concentration of the library was measured using Qubit 2.0. The library, library blank and PCR blank were run on Bioanalyzer to assess the presence of expected product of around 350 bp (Figures 15 - 17). Since the target of 350bp and all other unexpected fragment sizes appeared on the bioanalyzer result, XT was applied to cut out ca. 350 bp fragments of the library, as follows; the purified product were ran on the e-gel and cut out, then undiluted sample of the library was run on the Bioanalyzer after the e-gel (Figure 18). The final library was sent for sequencing.

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3.3.3. SEQUENCING

All libraries were sequenced using the illumina sequencing protocol in the University of Copenhagen, Copenhagen Denmark.



Figure 13: The Illumina HiSeq 2000 used for next generation sequencing at the University of Copenhagen laboratory in Denmark.

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3.3.4. ANALYSIS

Full details are not provided since the analyses were done by the Bioinformatics technician, however the COI barcode sequences from different taxonomic levels (Actinopterygii, Arachnida, Branchiopoda, Chilopoda, Insecta, Isopoda, Ostracoda, and Sarcopterygii) were downloaded from the BOLD v3 (Ratnasingham and Hebert 2007) Public Data Portal to create a database and its corresponding taxonomy map. Furthermore, the sequences of the eleven pest insects that were sequenced in this study were added to the database. Species identification was made with QIIME v1.7.0 (Caporaso *et al.* 2010). The work done by the technician was acknowledged.

From the results provided by the technician further analysis were carried by the researcher with the assistance from the mentors (Supervisors: Prof Taylor, Prof Gilbert and Dr Nangammbi and also assisted by other researchers which were acknowledged). The analysis includes the occurrences of Hemiptera and that of all families forming part of the diet. All the results were imported in Microsoft Excel where statistical analyses were carried out; tables and bar graphs are the results from Microsoft Excel spread sheets. It should be understood that in order to not overinflate the observed diversity of the sequences (which can happen due to errors appearing in the PCR and sequencing), all the faecal samples were amplified with at least duplicate PCRs. We then only retained sequences for analysis that were found identical in both PCRs that way you eliminate background error.

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Table 8: Calculation of adapter to be used for the ligation procedure: 10 X adapter used in order to make sure that an excess is added (adapter 25 uM stock):

	Emmanuel's purified PCR-pool
Concentration	19.9 ug/ml = 19.9 mg/l = 0.019 g/l
Length	227 bp
Molecular weight (M)	660 g/mol/bp x 227 bp = 149820 g/mol
Mol. conc.	(0,019 g/l) / (149820 g/mol) = 1.328 x 10-7 M (x 1000 x 1000) = 0.133 uM
Adapter concentration aimed at (10x)	1.33 uM
Dilute adapter 2x (25 uM adapter)	25/2 = 12.5 uM
Dilute in mix (1:10)	12.5 uM/10 = 1.25 uM
	5 ul adapter 25 uM + 5 ul H2O = 10 ul 1.25 uM adapter (when 5 ul of the adapter-mix is added to sample + library blank).

3.4. RESULTS

3.4.1. EXTRACTION AND AMPLIFICATION

PCR products were only considered positive when two products from two different combinations of primer pairs amplified. The results of PCR after gel electrophoresis were recorded and shown in Appendix 2; the samples with 0 µl used for library pool preparation are the ones which failed to produce two positive PCR products and were considered the failed samples (all of them were retried with different primer pairs and failed to amplify) and they were all eliminated from this stage. The primer pairs used for each and every sample are also indicated in the Appendix 2.

3.4.2. LIBRARY BUILD AND SEQUENCING

The band brightness was used to determine the volumes of the sample to be pooled; these volumes used are shown in Appendix 2. The pool concentration on Qubit HS was 99.3 ng/ml = 19.9 ug/ml hence to run on bioanalyzer the library was diluted.

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The size of nucleic acids in the pool determined by running the pool in the standard gel (Bioanalyzer) is shown in the eletropherogram in Figure 14 below.

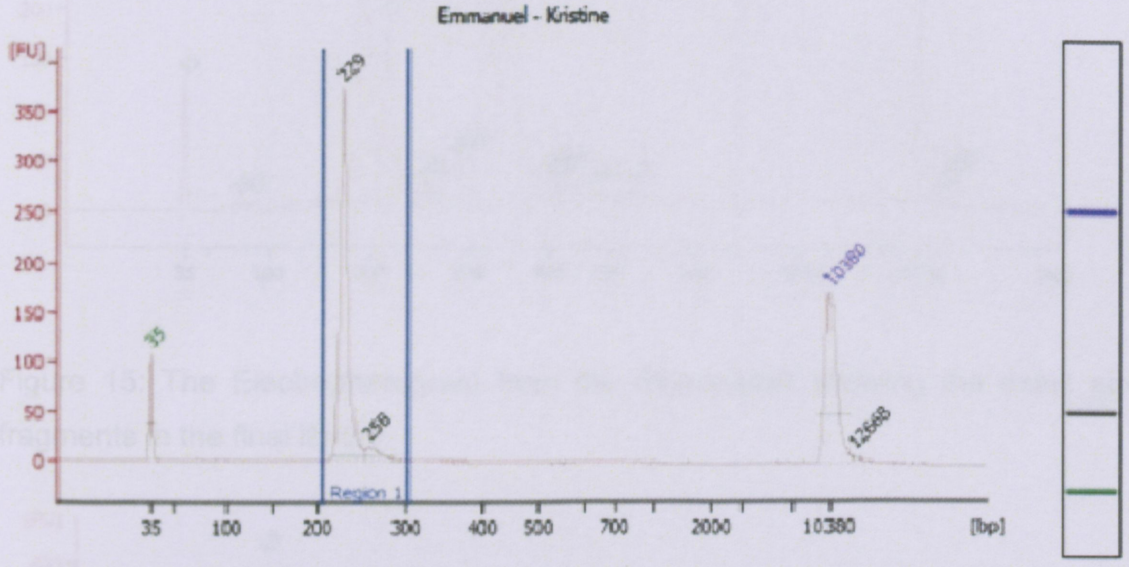


Figure 14: The Electropherogram from the Bioanalyzer showing the exact sizes of DNA fragments in the PCR pool.

From the PCR pool which seemed clean as in the figure above, the library was blunted and ligated with adapters and then filled-in with enzymes successfully. The library was indexed and amplified by running PCR. The PCR of the library (which was divided into five tubes) was positive. Librabry blank and PCR negative control was used where necessary to control the effectiveness the processes. The concentrations of the libraries on Qubit high sensitivity were as follows; the library was 37.3 ug/ml = 160.1 nM and hence was diluted; the library blank was 0.71 ug/ml; whereas the PCR blank was 0.574 ug/ml. All the products were run on Bioanalyzer and expecting fragment size of 350 bp for the Library. Figures 15-17 shows the results of the Bioanalyzer. The library was having so many sizes of their nucleic acids and was cut on XT and the results are shown in Figure 18. The library was then sent for sequencing.

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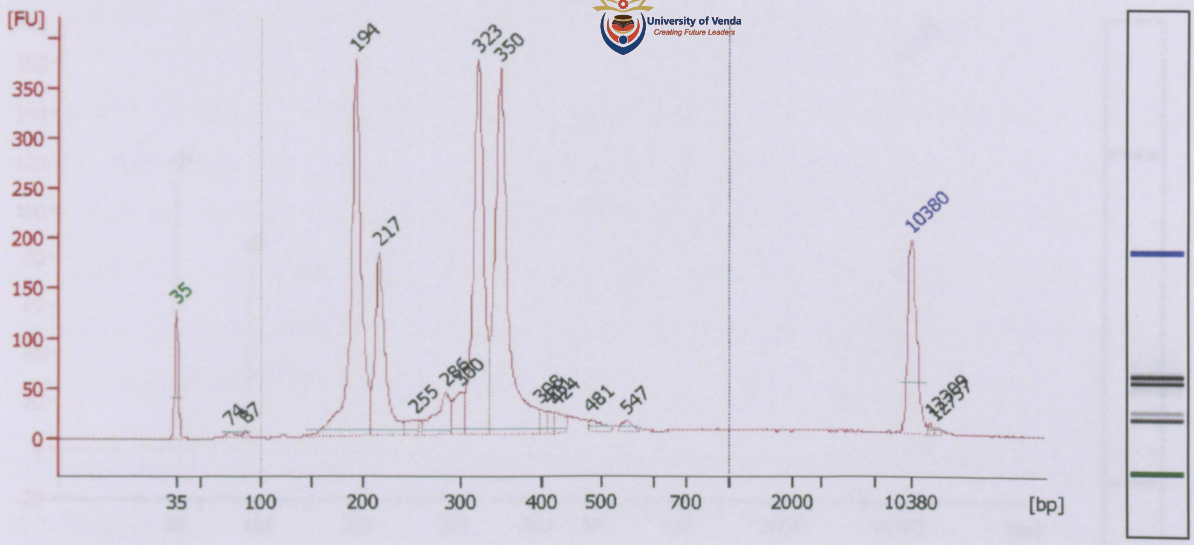


Figure 15: The Electropherogram from the Bioanalyzer showing the exact sizes of DNA fragments in the final library.

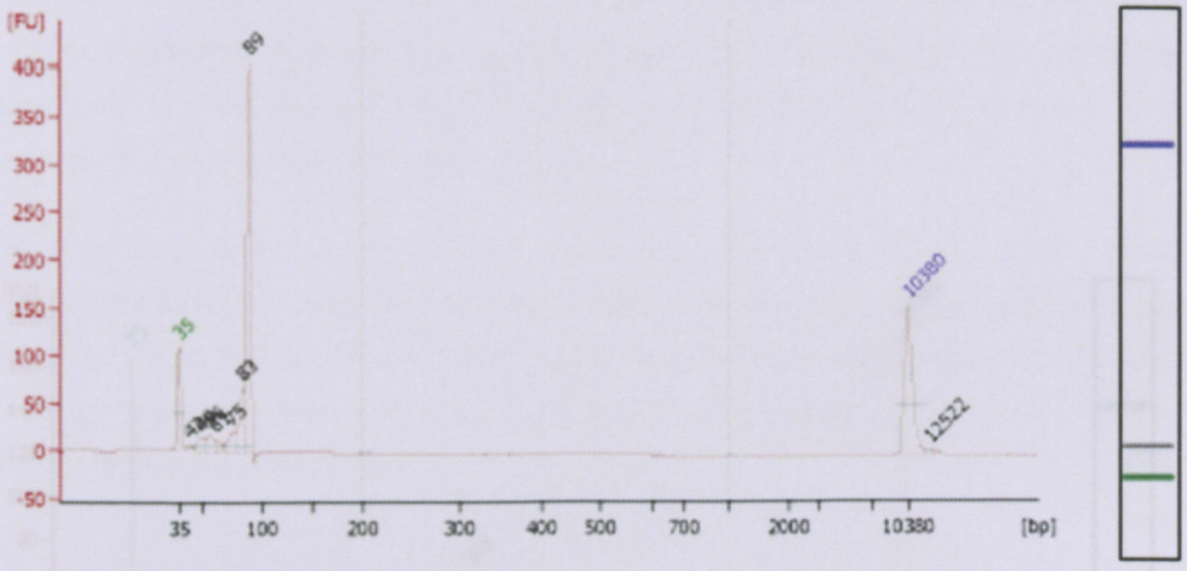


Figure 16: The Electropherogram from the Bioanalyzer showing the exact sizes of DNA fragments in the Library blank

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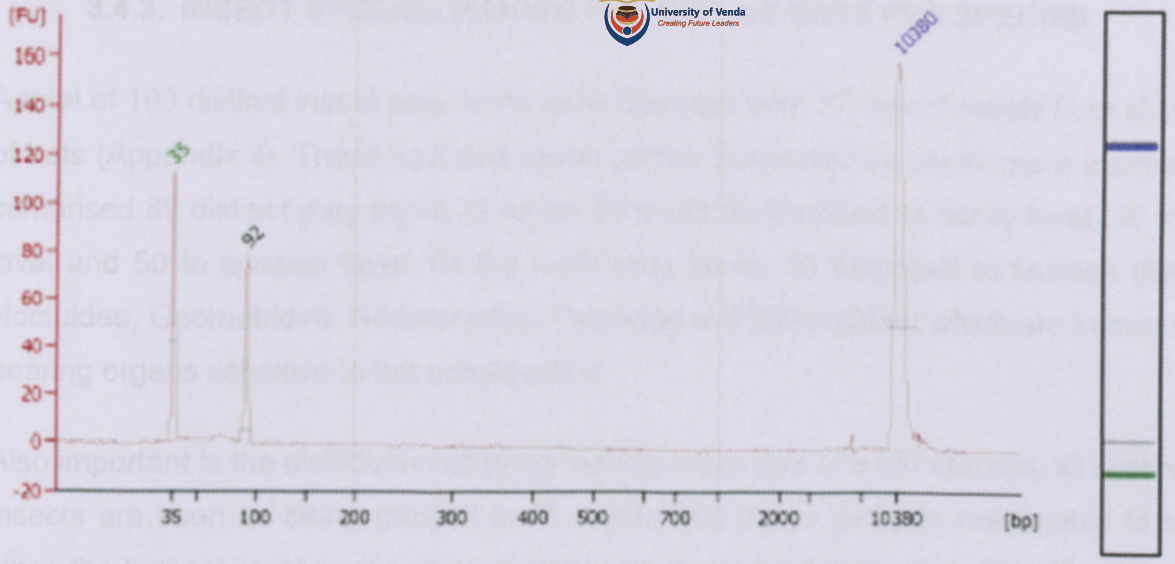


Figure 17: The Electropherogram from Bioanalyzer showing the exact sizes of DNA fragments in the PCR blank

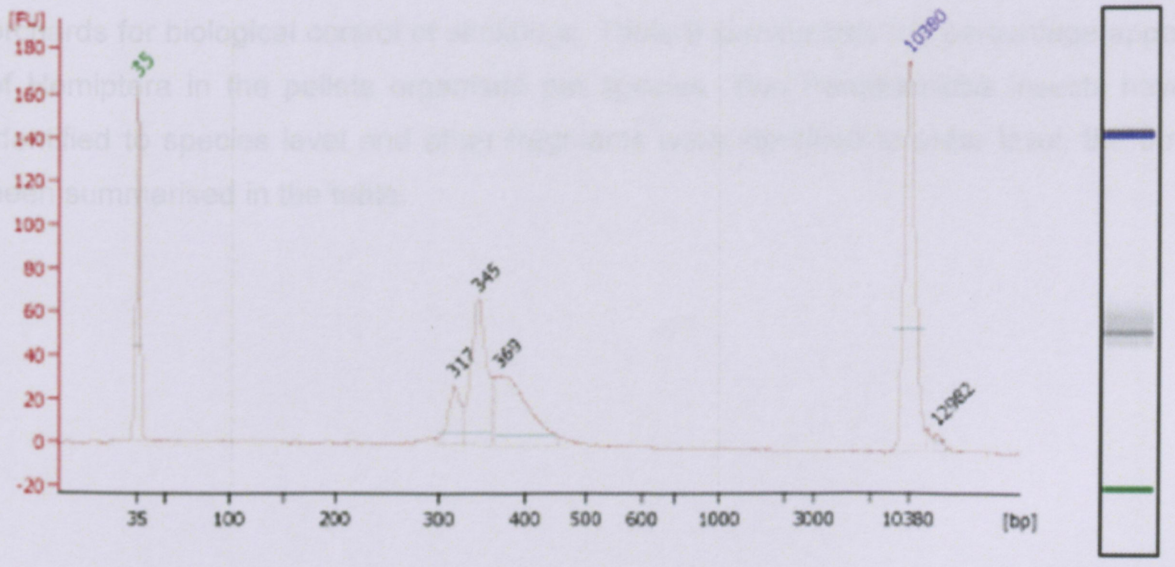


Figure 18: The Electropherogram from the Bioanalyzer showing the exact sizes of DNA fragments in the library after cut on XT

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3.4.3. INSECT SPECIES MAKING THE DIET OF BATS PER SPECIES

A total of 103 distinct insect prey items were detected from 37 faecal pellets from six species of bats (Appendix 3). These included seven orders dominated by Lepidoptera (moths) which comprised 89 distinct prey items, of which 89 could be identified to family level, 86 to genus level and 50 to species level. Of the moth prey items, 66 belonged to families (Erebidae, Noctuidae, Geometridae, Notodontidae, Pyralidae and Sphingidae) which are known to have hearing organs sensitive to bat echolocation.

Also important is the distribution of these insects in the diet of a bat species, all orders of this insects are seen as being present in *M. midas* and this is perhaps reasonable to see this since the highest number of pellets analysed are from this bat species. The *M. midas* seems too dominated by Order Lepidoptera.

Although few samples have been used for other bats in this study there is a remarkably high variation number of insects' in the diets. The lowest is the diet variations of *H. caffer* which is dominated by Lepidoptera followed by Coleoptera forming only approximately 8 %; this bat has recorded only insects from two orders.

The main focus of this study was initially to find which bats can be attracted to macadamia orchards for biological control of stinkbugs; Table 9 summarizes the percentage appearance of Hemiptera in the pellets organised per species. Two Pentatomidae insects have been identified to species level and other fragments were identified to order level, the three has been summarised in the table.

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Table 9: Summary of results from second generation sequencing for Hemiptera prey

				% occurrence (no. of pellets) of Hemiptera		
Species	Sample months	No. prey Orders	No. pellets analysed	***N. viridula***	***M. acuta***	Hemiptera (not identified)
N. thebaica	May 2012, Aug 2010, Aug 2012	5	6	16.7	16.7	16.7
S. dinganii	Feb 2011, Feb2012	5	2	100	50	100
P. hesperidus	Aug 2011, Jan 2012	6	3	66.7	0	66.7
C. pumilus	Apr 2012	5	1	100	100	100
H. caffer	Aug 2012	2	1	0	0	0
M. midas	Aug & Nov 2012	7	24	25.0	16.7	29.2
Total			37	32.4	18.9	35.14

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3.4.4. PROPORTIONS OF INSECT ORDERS IN BAT DIET BASED ON NGS BY OCCURRENCE AND "VOLUME" (PROPORTION OF GENE FRAGMENTS)

The proportional occurrences of insect orders per bat species are shown in Figure 19 (percentage of number of pellets having the order divided by the total number of pellets analysed per species).

The proportion of gene fragments of each order (expressed as the total of fragments for each bat species) are shown in Figure 20 (ratios of number of fragments identified for each insect order divided by the total number of fragments identified for the specific bat species).

M. midas is the only bat with large number of pellets samples, only the diet variations of *M. midas* was analysed separately for date and for locations by insect order and family and shown in Figure 21 a) to c). The figures present data as percentage occurrences (number of pellets with that family divided by the total number of pellet samples per species)

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Figure 19: The proportions of different insect orders in the diet of 6 genera of bats occurring in macadamia orchards based on the frequency of occurrence (number of pellets containing that order divided by the total number of pellets in the species sample) based on NGS results of this study. Sample sizes (n) represent the number of pellets.

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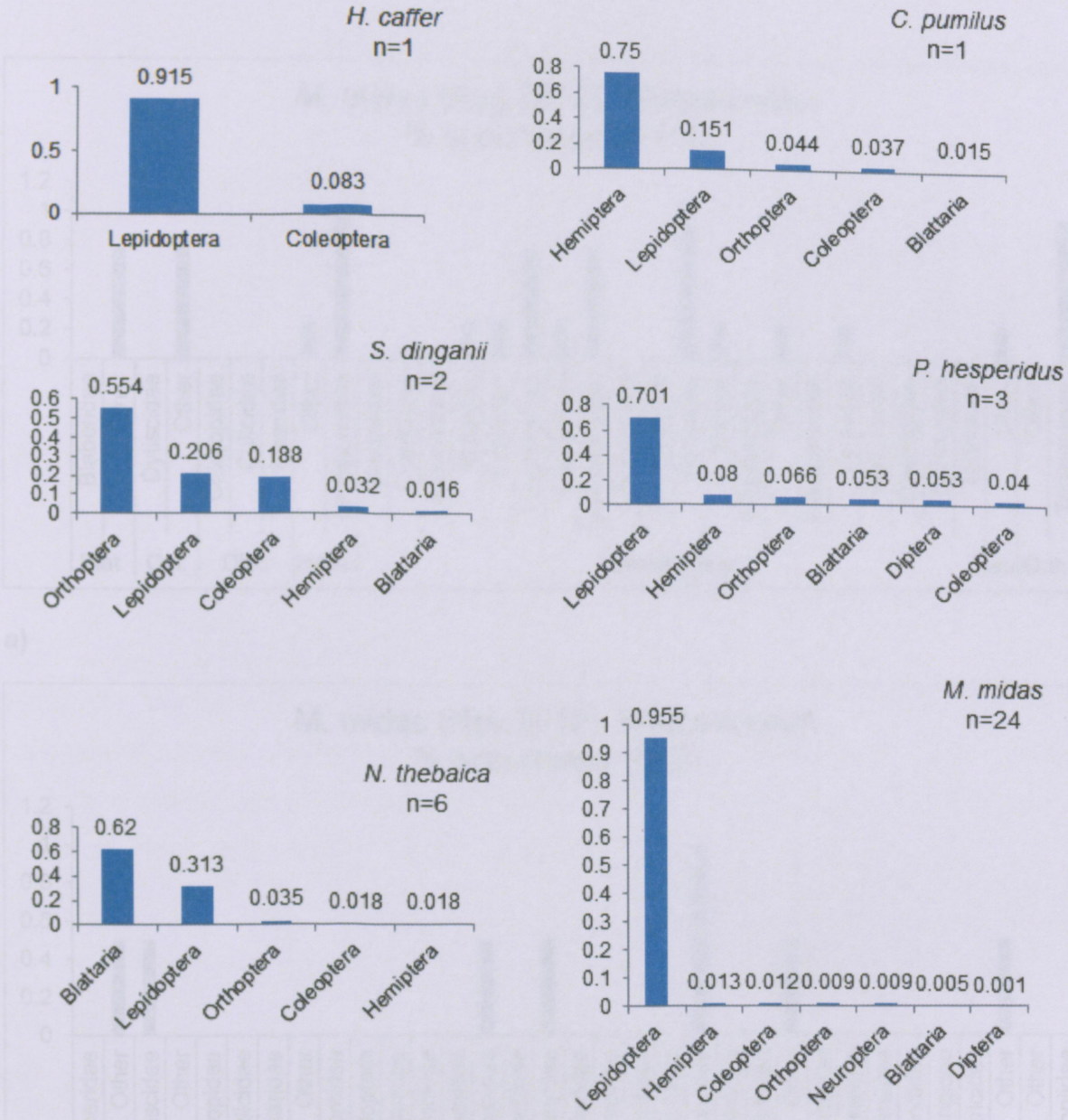
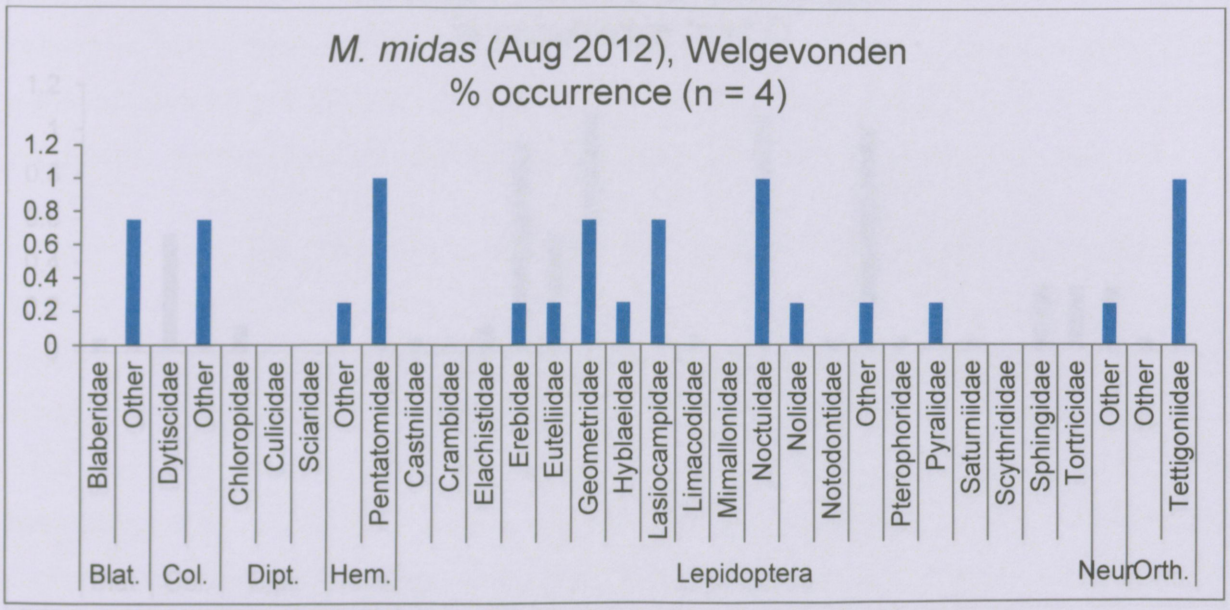
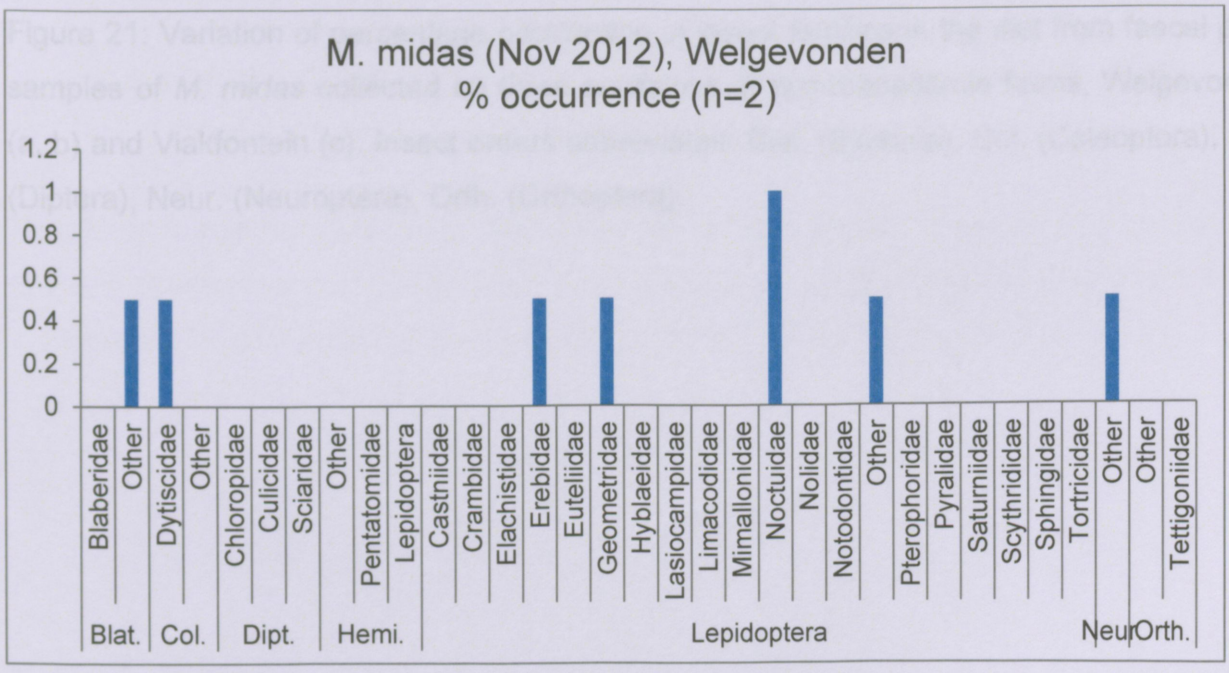


Figure 20: The proportions (by number of gene fragments, "volume") of different orders of insects in the diet of six genera of bats based on NGS results of this study. The number of gene fragments represented by a single order for a particular species was divided by the total number of gene fragments isolated from that species. The samples sizes (n) refer to the number of pellets per species sample.

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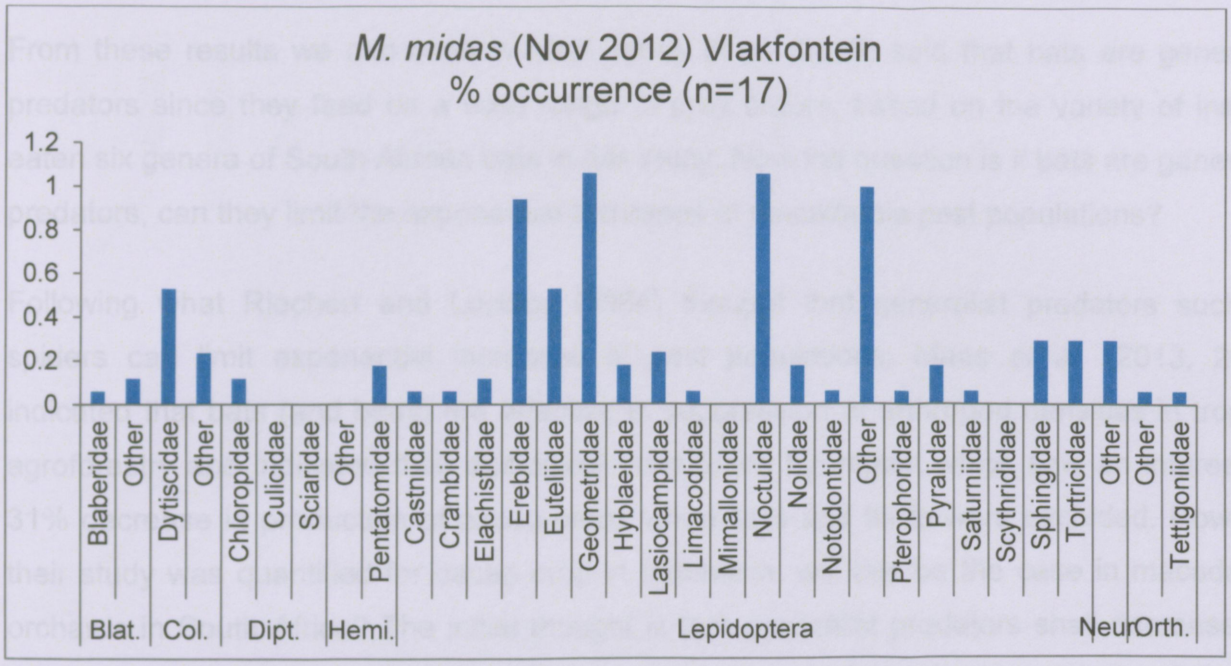


a)



b)

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c)

Figure 21: Variation of percentage occurrence of insect families in the diet from faecal pellet samples of *M. midas* collected on three occasions at two macadamia farms, Welgevonden (a, b) and Vlakfontein (c). Insect orders abbreviated: Blat. (Blattaria), Col. (Coleoptera), Dipt. (Diptera), Neur. (Neuroptera), Orth. (Orthoptera).

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From these results we can confirm what Jones *et al.* (2009) said that bats are generalist predators since they feed on a wide range of prey orders, based on the variety of insects eaten six genera of South African bats in this study. Now the question is if bats are generalist predators, can they limit the exponential increases of macadamia pest populations?

Following what Riechert and Lockley (1984) thought that generalist predators such as spiders can limit exponential increases in pest populations, Maas *et al.* (2013, 2015) indicated that bats (and birds) are effective in suppression of arthropod densities in tropical agroforestry and indicated their ecological importance by impact which saw an impressive 31% decrease in production of cacao crops when bats and birds were excluded. However their study was quantified for cacao crop in Indonesia, will this be the case in macadamia orchards in South Africa? The initial thought is that generalist predators shall decrease the arthropod density thereby being effective biological control agents, and the following discussion is based on that.

3.6.1. THE COMPARISON OF NEXT GENERATION SEQUENCING AND CONVENTIONAL MICROSCOPIC METHODS

Results from microscopic faecal dissection (for three bat species included of in this study and one species not part of this study) were provided by Taylor *et al.* (2013) and are shown in Figure 22 for comparison with the molecular data (Figures 19 to 21 above).

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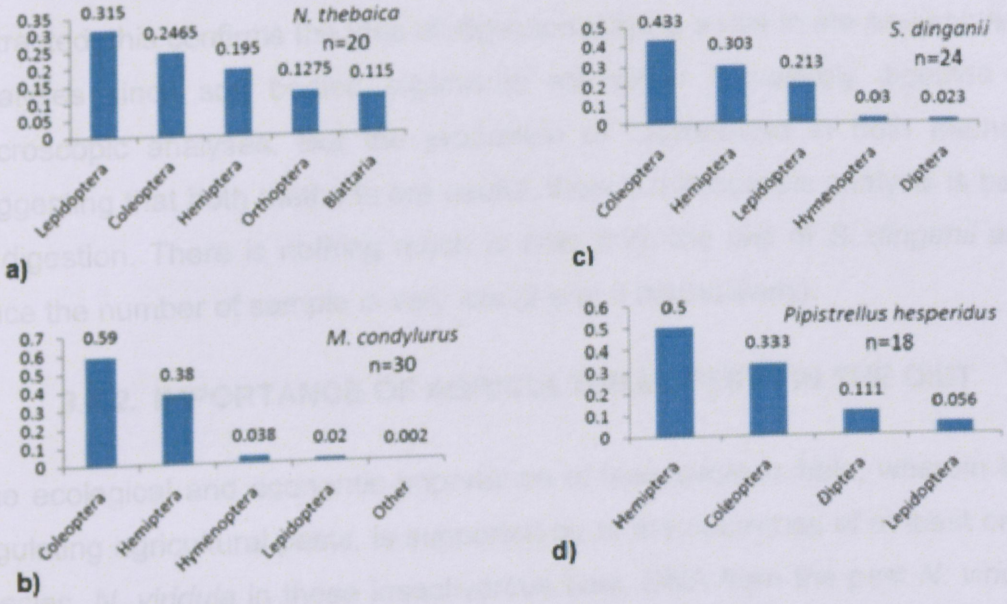


Figure 22: The proportions (by volume) of different orders of insects in the diet of four genera of bats occurring in macadamia orchards based on microscopic dissection of faecal pellets (from Taylor *et al.* 2013). Sample sizes (n) represent the number of pellets for each species.

Looking at the pellets analysed microscopically the sample size is reasonable and should be enough to conclude the diet of the specific bat species, but this can be directly affected by the fact that microscopic analysis can be used to accurately estimate the diet of the bats to family level (Taylor *et al.* 2013). NGS provides much greater taxonomic resolution but is very expensive and may not give reliable estimates of the volume of different prey items, due to possible biases in PCR-amplification of different insect taxa. The microscopic method is very cheap and can give a possibly more accurate estimation of the proportional volume of different prey items but only to Order or Family level.

It is therefore necessary to perform both methods on the same sample to get an indication of how the proportion of gene copies of different prey items obtained from NGS may be a realistic indicator of prey volumes as seen in microscopic analysis. The diet of *N. thebaica* was shown to be confirmed with both methods; other bats shown in microscopic analyses for comparison are *S. dinganii* and *P. hesperidus*. This can be shown by presentation of similar orders and families' proportions of results.

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The diet of *N. thebiaca* indicates that the soft bodied organisms form order Blataria dominate the molecular results as expected because the DNA of soft bodied organisms can easily be extracted; this confirms the idea of digestion playing a role in the presentation of microscopic analyses since soft bodied organisms are either completely digested or unidifieble in microscopic analyses. But the proportion of Lepidoptera in both methods is the same suggesting that both methods are useful, though microscopic analysis is based on left overs of digestion. There is nothing much to note from the diet of *S. dinganii* and *P. hesperidus* since the number of sample is very low (2 and 3 respectively).

3.6.2. IMPORTANCE OF AGRICULTURAL PESTS IN THE DIET

The ecological and economic importance of insectivorous bats, wherein bats are useful in regulating agricultural pests, is supported by of the ocurrence of at least one significant pest species, *N. viridula* in these insectivorous bats. DNA from the pest *N. viridula* was found in feaces of five of the six species of bats sampled. Apart from *N. viridula*, other pest species were identified in the bats diet. (E.g. Mythimna is a pest of sugar cane and cockroaches (Blattaria) are household pests etc.). Although Torticidae moth species (*Cochylidia implicitana* and *Platphalonidia felix*) were included in the diet of *M. midas* these are not pest species.

Failure to obtain reference sequences of *N. pallidoconspersa* and *B. natalicola* and the species absence on GenBank or BOLD resulted in an inability to establish whether unidentified hemipteran sequences in our sample could perhaps belong to these two pest species.

3.6.3. THE DIET OF THE BATS

Samples of faecal pellets were small in all species except for *M. midas*. Dietary studies have been performed in all the species included in this study except for *M. midas*. Results of this study were published in part in the Southern African Macadamia Growers' Association (SAMAC) (Taylor *et al.* 2013; Appendix 4)

3.6.3.1. The diet of *P. hesperidus*

P. hesperidus is a clutter-edge forager, and its diet includes Coleoptera, Hemiptera, Diptera and Lepidoptera (Schoeman 2006). Molecular analyses of the diet of *P. hesperidus* were based on 3 pellets which are from a single organism and this implies that the results cannot

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be so conclusive, but it is enough to suggest the possible diet of that species at that particular season of the year.

Appendix 3 below shows 35 insects from six orders and 17 families which have been found in the diet of this bat. When calculated for percentages (occurance of insect orders per pellet), Lepidoptera has been dominating in all pellets, followed Coleoptera, Hemiptera, Diptera and Orthoptera which appeared in two of the three pellets whereas Blattaria occurred in only one pellet.

N. viridula was found in the diet of *P. hesperidus*hence it can be a suggested that if more pellets of *P. hesperidus*can be studied it can give more details on its impact on *N. viridula* and other pest insects. This evidence was used to bring the assumption that *P. hesperidus*can be a good biological control agent of pest insects in macadamia orchards.

Also from the microscopic analysis where 18 pellets have been used (suggesting more representative results), 50% of *P. hesperidus*diet was composed of Hemiptera (not limited to Pentatomidae) which is a good sign too that this bat species can be a good control agent. The detailed results of the microscopic analysis were not part of this study but It is thought to assist and if combined to molecular results it will give an approximately 29% chance that the Hemiptera can be in the diet of *Pipistrellus hesperidus.*

It is of high significance to mention that results from this study suggest that *P. hesperidus*is the second most variable bat species feeding in six of seven insect orders found in this study, not forgetting to say 17 families of these insects found in its diet.

3.6.3.2. The diet of M. midas

Monadjem *et al.* (2010) classified this bat as an open-air forager. Based on its robust skull, mandible, and dentition, including the data from stomach contents, it is suggested that it feeds predominantly on Coleoptera (Archer 1977). However apart from the study by Archer (1977) nothing has been documented in this species

From this study only this bat is feeding from seven insect orders dominated by Lepidoptera with 89 families in its diet. These orders are Lepidoptera, Coleoptera, Hemiptera, Diptera, Orthoptera, Blattaria, and Neuroptera.

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The details of the outcomes of this bat have been discussed in the next sub section below

3.6.3.3. The diet of N. thebiaca

Monadjem *et al.* (2010) classified this bat as a clutter forager with a diet that varies seasonally, with Orthoptera, Coleoptera and Lepidoptera dominating in most areas, but also confirmed that the bat takes a variety of other insects and arachnids in small proportions.Schoeman and Jacobs (2003) analysed 51 fecal pellets from 8 bats of *N. thebiaca* and found that the diet to be comprised of seven insect orders which are Lepidoptera (Moths and Butterflies), Coleoptera (Beetles), Hemiptera (True Bugs), Diptera (Flies), Hymenoptera (Bees, Wasps and many more), Orthoptera (Locusts and Crickets), and Arachnida (Spiders, mites and many more). Taylor *et al.* (2012) described the insect orders of the culled parts to nine insect orders adding Ephemeroptera (Mayflies), Blattaria (Cockroaches) and Mantodea (Mantis) bringing the number of insect orders which this bat feeds on to ten.

This study analysed only six pellets and found five insect orders from the list above. This is also confirmed in the study by Taylor *et al.* (2013) which found four of the five insect orders. All this studies shows that this bat does eat Hemiptera and *N. virudula* specifically and hence it can be used as pest control agent in macadamia orchards.

3.6.3.4. The diet of S. dinganii

Based on microscopic analysis of ten samples Jacobs and Barclay (2009) found that *S. dinganii* feeds on four insect orders, Coleoptera (Beetles), Lepidoptera (Moths and Butterflies), Hemiptera (True Bugs), and Hymenoptera (bees, wasps and many more). The microscopic analyses provided by Taylor *et al.* (2013) shows that this bat species does feed on insects described by Jacobs and Barclay (2009).

From two samples used in this study *S. dinganii* feeds on five insect orders including three that Jacobs and Barclay (2009) has described that's adding the total of insect orders to six by Orthoptera and Blattaria. Specifically we also recorded *N. viridula* in the diet. The combinations of these studies suggest that *S. dinganii* feeds on insects from seven orders. The order which is dominating is Orthoptera (grasshoppers and crickets) in this study. This order is also important in the biological control of stinkbugs in macadamia orchards since it does feed on *N. viridula* which are the major pest insects in macadamia.

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3.6.3.5. The diet of C. pumilus

Bohmann *et al.* (2011) found a total of six insect orders, which includes Coleoptera (Beetles), Diptera (Flies), Hemiptera (True Bugs), Isoptera (Termites), Lepidoptera (Moths and Butterflies), and Trichoptera (Caddis flies), containing 25 families within the diet of *C. pumilus*. Monadjem *et al.* (2010) said *C. pumilus* is an open-air forager which feed on Coleoptera, Hemiptera, Lepidoptera, Hymenoptera and Diptera.

It is important that the diet of *C. pumilus* varies greatly with respect to location of feeding; this is because in Kenya and Kruger National Park, South Africa the diet of *C. pumilus* is mainly Hemiptera and Coleoptera with some Lepidoptera (Aldridge and Rautenbach 1987; Whitaker and Mumford 1978) but elsewhere in Kenya at Lake Naivasha, more than 50% of the diet is Diptera with Coleoptera and Hemiptera eaten to a lesser extent (Clark and Smart 1991), and at Amani in Tanzania, Blattaria is the major insect order in the diet of *C. pumilus* with Hemiptera being the only other insect order (Aspetsberger *et al.* 2003).

Of the six families identified in this study, three of them formed part of the results for Bohmann *et al.* (2011) study which are Coleoptera, Hemiptera, and Lepidoptera though Bohmann *et al.* (2011) was a Swaziland study, combined with this study they suggest that *C. pumilus* is responsible for consumption of eight insect orders and combined with Monadjem *et al.* (2010) they add the tally of insect orders to nine.

Only one sample has been analysed for this species and all the Hemiptera forming part of bats diet in this study have been found, though it might be by chance the sample was collected in April when the stinkbugs peak has already passed and in Bohmann *et al.* (2011) Hemiptera was found to be part of the diet of this bat. This suggest that this bat can be a control agent of Hemiptera in macadamia orchards but this needs to be confirmed first once all the barcoding sequences of more Hemiptera pests are available in GenBank.

Our study suggest that *C. pumilus* feeds mostly on Hemiptera which is not the case in Bohmann *et al.* (2011) were 30 fecal pellets suggested that Hemiptera comes third in propotions after Lepidoptera and Diptera respectively. However this is reasonable since only one pellet was analysed for this study.

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3.6.3.6. *The diet of H. caffer*

Aerially-foraging insectivorous bats use echolocation for orientation and/or to locate their prey. When hunting, these bats often emit intense pulses of sound (>110 dB) and analyse the much fainter echoes reflected by objects to detect, localise and possibly classify potential prey (Schnitzler and Kalko 2001). By this the bat is a moth specialist and feeds mostly on this insect, and Monadjem *et al.* (2010) classified this bat as a clutter forager with the diet consisting predominantly of Lepidoptera, with smaller quantities of Trichoptera and Coleoptera.

This study confirms the dominance of Lepidoptera in the diet of this bat species, followed by Coleoptera. There are several other studies confirming that this bat is a specialist and doesn't feed on a wide variety of insect (Schoeman 2006; Aldridge and Rautenbach 1987).

Also to note importantly is the absence of Hemiptera, the major pest insects in the macadamia orchards, and the absence of Tortricidae moths which are the other pests of macadamia, suggesting that this species is unlikely to be successful as a biological control of pests in macadamia orchards. But only 1 fecal pellet was analysed so clearly it is premature to say this!

3.6.4. VARIATION IN THE DIET OF *M. MIDAS*

Since only this species has a high number of pellets analysed it was great interest to check the variation of diet with respect to date and location. The number of pellets collected in Welgevonden (6) was very low as compared to 17 in Vlakfontein, and as expected it reflects on percentage occurrence of insects.

Salinas-Ramos *et al.* (2015) believes that the availability and abundance of insect prey influences the diet of insectivores and this can be lead to variation on diet either seasonally or annually (Clare *et al.* 2014). Though the sample size is not enough to make variation comparisons, the following few variations in *M. midas* can be noted with respect to date in November 2012 twenty seven insect families from all seven orders were found in the diet of *M. midas* in both locations combined which is very high compared to the insects families found on August which is only fifteen from six families.

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The higher number of insect families provides evidence that this bat is a generalist insectivore, but important variation seen here is the absence of Diptera in the diet in August which is supported by Clare *et al.* (2014) who found out that the presence of Diptera in the diet of *Eptesicus fuscus* decreases in the late season of the year, Fukui And Agetsuma (2010) also confirmed that bat diet has variation based on the availability of food.

Because of less number of pellets for Welgevonden farm not much can be seen as major variations in the diet of this bat. Only two pellets were collected in November which makes the conclusion of variation difficult because the variation can be because of the date at which the pellets were collected at.

3.7. CONCLUSIONS

NGS data from bat fecal pellets had confirmed the known diet of six South African bat species based on previous microscopic dietary studies and has provided a greater taxonomic precision of prey items eaten down to family, genus and species level depending on availability of reference sequences on GenBank and BOLD.

The slow-flying stink-bugs have been found in the diet of open-air feeders (*M. midas* and *C. pumilus*), clutter-feeding bats (such as *N. thebaica*) and clutter edge bats (such as *P. hesperidus*and *S. dinganii*) and because of sample size none of Hemiptera has form part of clutter-feeding bat *Hipposideros caffer*.

Since moths are known to be prevalent in the diet of most of the bats included in this study and previous researches has shown that the moth macadamia nut borer peaks in December and January, this moth is predicted to be present only in bat fecal pellets collected in December and January, and since most of our pellets were not collected around that time the absence of this pest moth is not surprising.

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Macadamia are attacked by indigenous pests which include moth species, stinkbugs and thrip species, affecting the success of industry in South Africa. Universally, stinkbugs (Hemiptera: Pentatomidae and Coreidae) have been identified as the major pest of macadamias, their damages in macadamia and avocado industries amounts up to R50 million annually. This led to recommendation of implementing integrated pest management (IPM). However the biological control of agricultural pests by both diurnal (birds) and nocturnal (bats) flying predators has been studied in most agricultural fileds.

To understand how can the pests be managed, two approaches were employed; 1) DNA of some of these pests was sequenced to obtain a reference library of DNA barcode sequences (in Chapter 2) and; 2) NGS of fecal pellets of 6 bat species was done to determine the diet of bats and evaluate potential importance of bats as consumers of pest insects in macadamias (in Chapter 3)

The study had the following hypotheses which were either supported or not supported as follows.

1. *Sequence data for the COI gene should allow correct identification of the hemipteran pest and non-pest species collected from macadamia orchards in this study, making DNA barcoding a useful tool for rapid identification of this major group of pests of the macadamia industry*

   As hypothesized, I found that barcoding was partly successful for identification, i.e. blast search matches of 85-99%, indicating the success of the COI Gene for identification. The intra-specific distances were smaller than the inter-specific distances also support the hypothesis for correct identification.

2. *Sequence data for the COI gene from hemipteran species and genera should provide a useful phylogenetic marker to allow an improved phylogenetic analysis of southern African bugs (Hemiptera) and in particular to test the monophyly of the two "stinkbug" families, Pentatomidae and Coreidae.*

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My data did not support that the COI gene sequence data were useful for phylogenetic analysis because statistical support for basal nodes was poor and did not recover monophyly in major stinkbug families, Pentatomidae and Coreidae

3. *NGS data from bat fecal pellets should corroborate the known diet of six South African bat species based on previous macroscopic dietary studies but provide greater taxonomic precision of prey items eaten down to family, genus and species level depending on availability of reference sequences on GENBANK and BOLD.*

My data has proved to corroborate with data described on previous researches and also published data form all over the globe. That suggest this hypothesis has been supported.

4. *The slow-flying stink-bugs should be more prevalent in the diet of clutter-feeding bats (such as N. thebaica and Hipposideros caffer) and clutter edge bats (such as P. hesperidusand S. dinganii) and less prevalent in open-air feeders like M. midas and C. pumilus .*

This hypothesis is not supported since all bats seem not only to be able to feed on stinkbugs which fly slowly near clutter but even on insects such as orthopterans (crickets) and Blattaria (cockroaches), suggesting that the idea that bats are generalist and feed on wide spectorum of bats.

5. *Since moths are known to be prevalent in the diet of most of the bats included in this study and previous researches has shown that the moth macadamia nut borer peaks in December and January, this moth is predicted to be present only in bat fecal pellets collected in December and January.*

My data suggest the absence of this moth, this maybe because of low sample size and sampling dates since bats tend to feed on more insects during seasons when the insect prey abundance is low.

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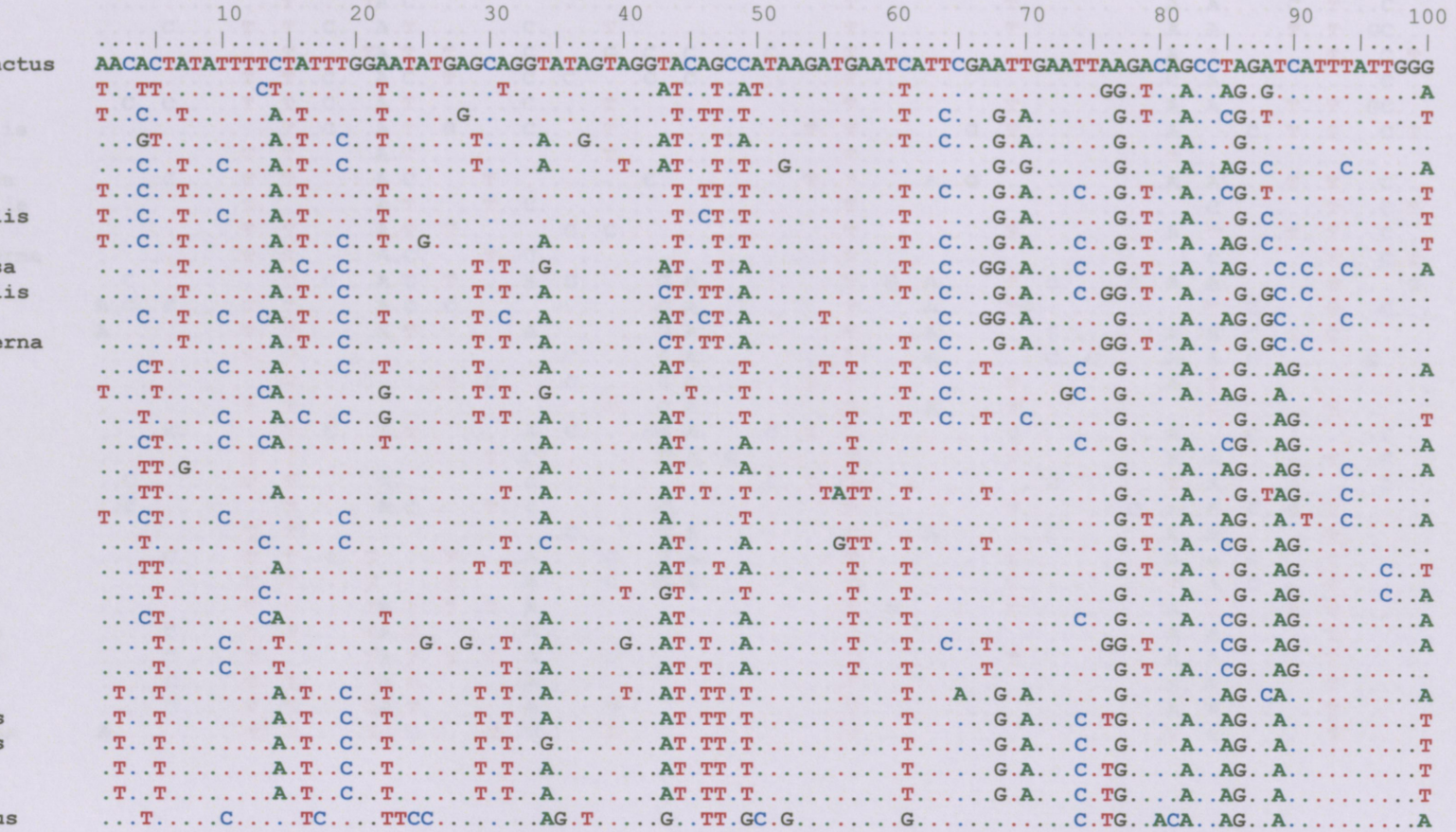
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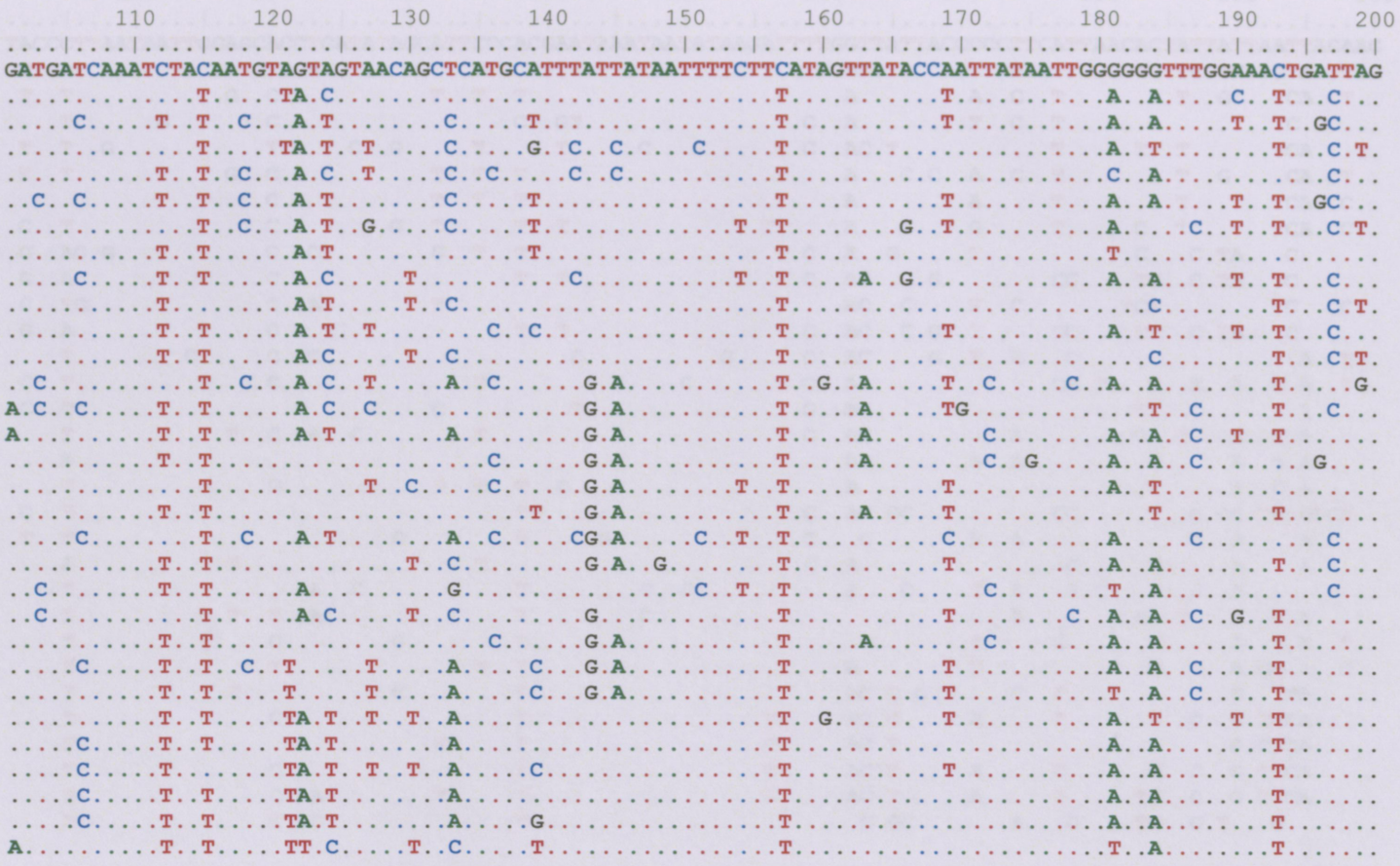


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APPENDIX 1: SEQUENCES USED FOR ANALYSIS

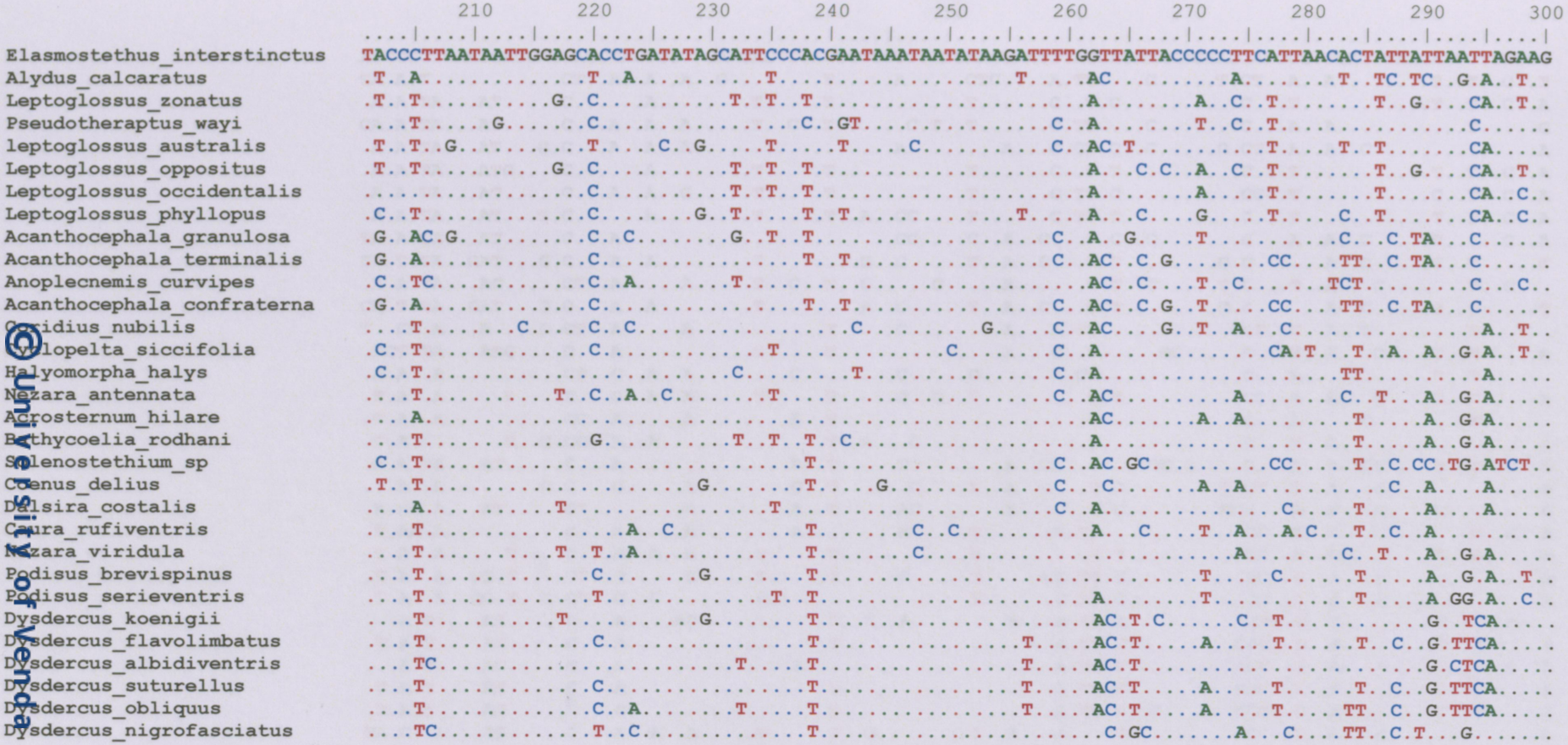


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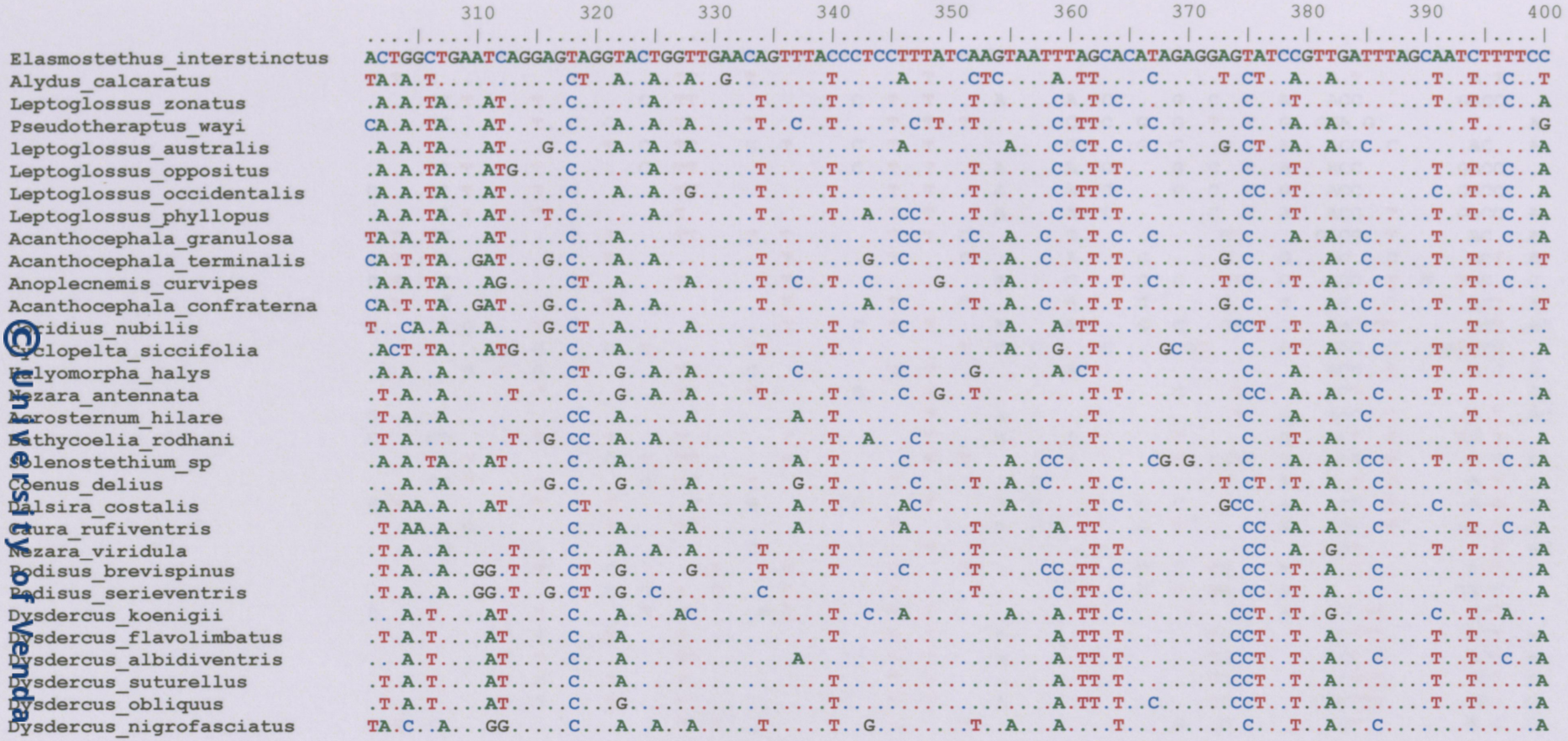
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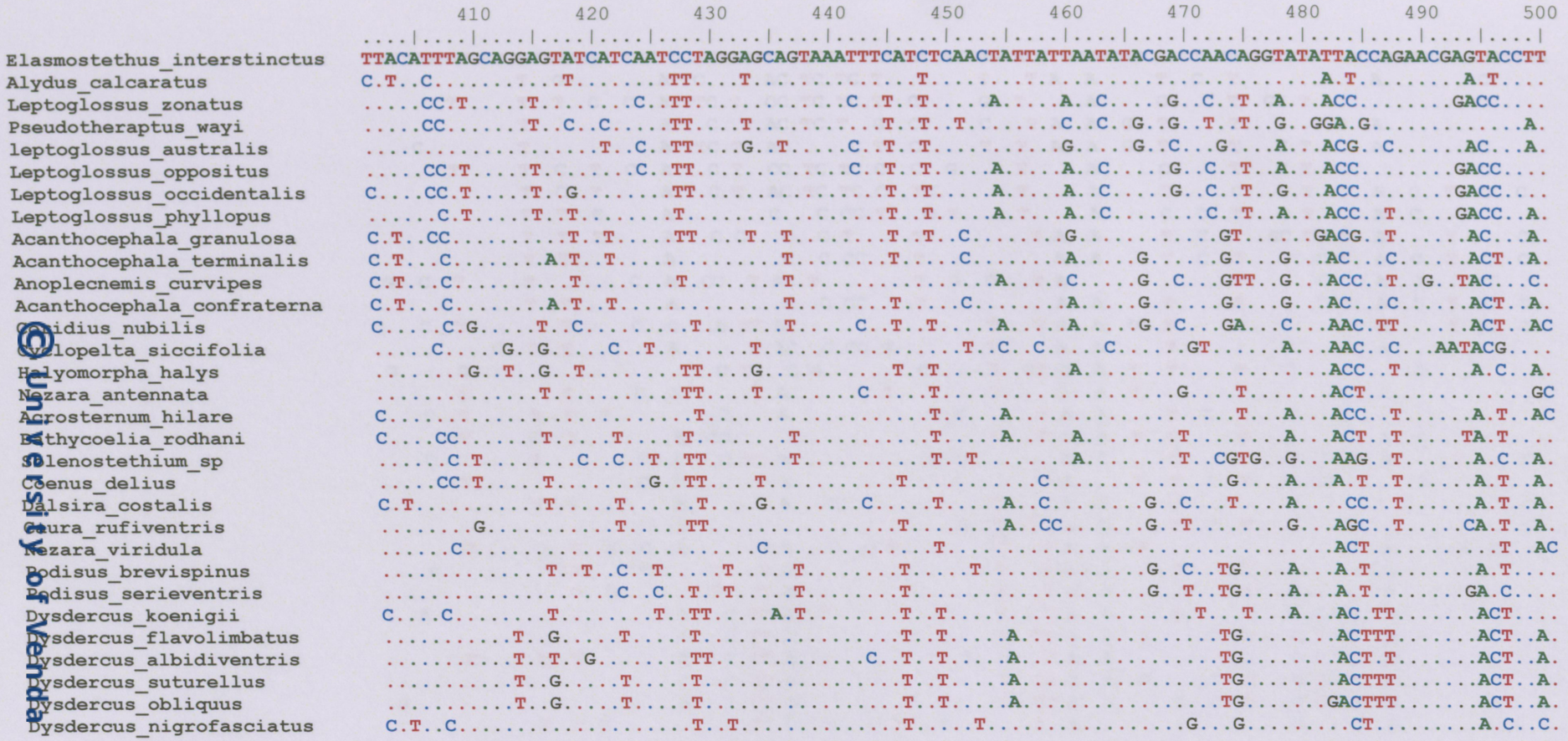


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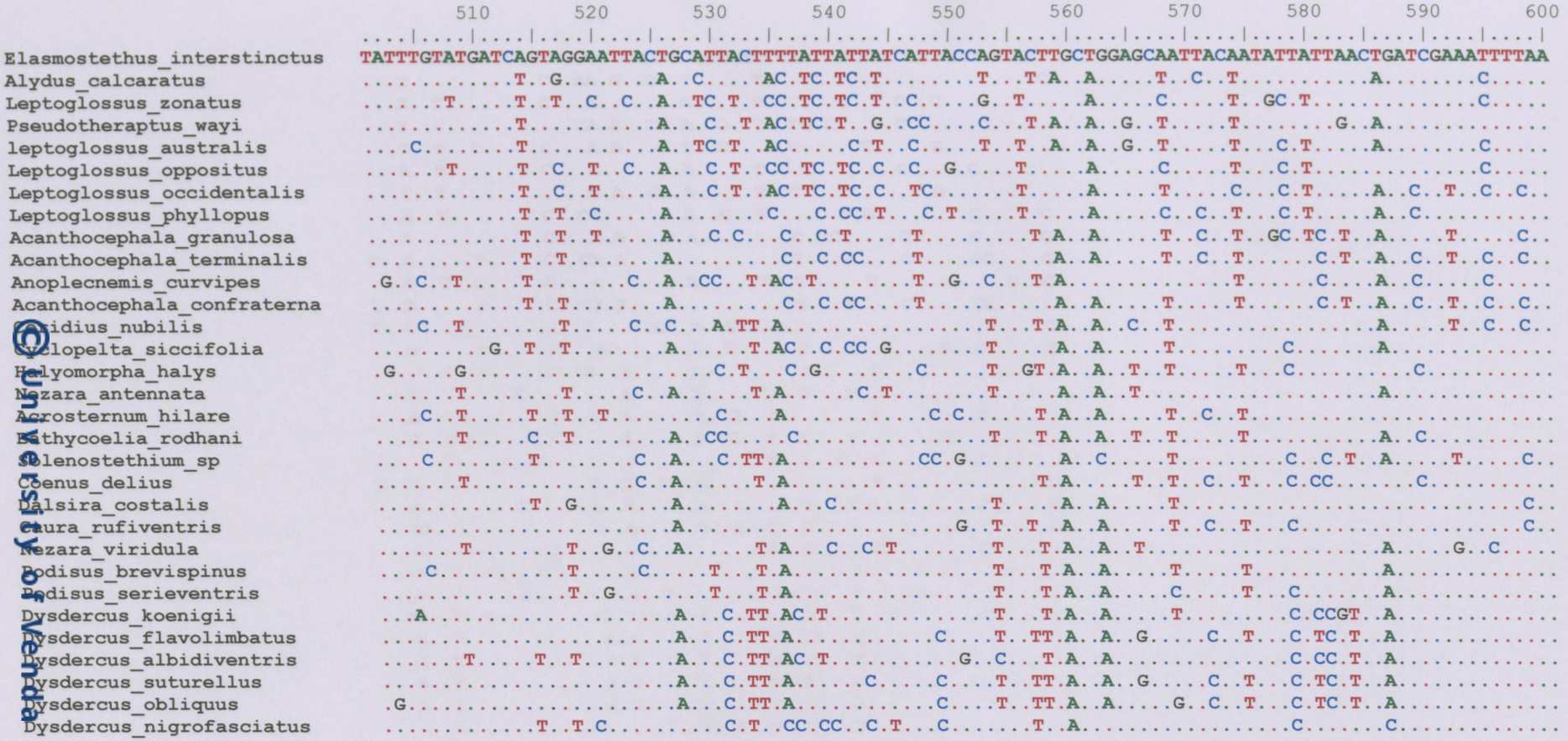
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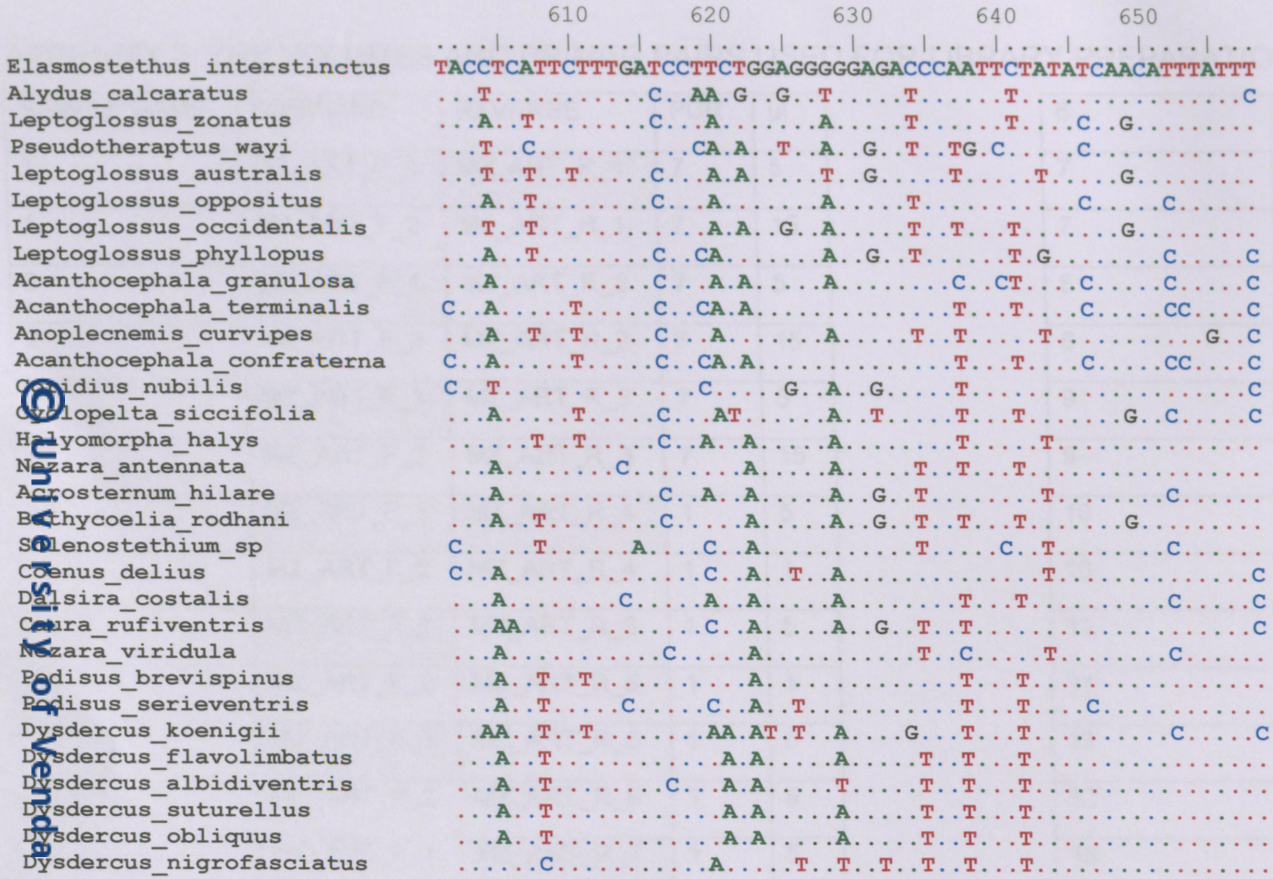


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APPENDIX 2: THE VOLUMES AND PRIMER PAIRS USED FOR LIBRARY PREPARATION

SAMPLE NAME	FORWARD	REVERSE	PCR	µl
1	MZ_ART_F_1	MZ_ART_R_1	7	5
1	MZ_ART_F_2	MZ_ART_R_1	7	15
2	MZ_ART_F_1	MZ_ART_R_2	7	5
2	MZ_ART_F_2	MZ_ART_R_2	7	15
3	MZ_ART_F_1	MZ_ART_R_3	7	5
3	MZ_ART_F_2	MZ_ART_R_3	7	15
4a	MZ_ART_F_1	MZ_ART_R_4	1	5
4a	MZ_ART_F_2	MZ_ART_R_4	1	1
4b	MZ_ART_F_1	MZ_ART_R_5	1	5
4b	MZ_ART_F_2	MZ_ART_R_5	1	1
4c	MZ_ART_F_1	MZ_ART_R_6	1	5
4c	MZ_ART_F_2	MZ_ART_R_6	1	5
4d	MZ_ART_F_1	MZ_ART_R_7	1	5
4d	MZ_ART_F_2	MZ_ART_R_7	1	1
5	MZ_ART_F_1	MZ_ART_R_8	7	0
5	MZ_ART_F_2	MZ_ART_R_8	7	0
6	MZ_ART_F_1	MZ_ART_R_9	7	1
6	MZ_ART_F_2	MZ_ART_R_9	7	15
7	MZ_ART_F_1	MZ_ART_R_10	7	1
7	MZ_ART_F_2	MZ_ART_R_10	7	15
8	MZ_ART_F_5	MZ_ART_R_16	4	1
8	MZ_ART_F_4	MZ_ART_R_1	4	5
9	MZ_ART_F_3	MZ_ART_R_2	4	15
9	MZ_ART_F_4	MZ_ART_R_2	4	15
10	MZ_ART_F_3	MZ_ART_R_3	4	5
10	MZ_ART_F_4	MZ_ART_R_3	4	1
11	MZ_ART_F_1	MZ_ART_R_11	7	1
11	MZ_ART_F_2	MZ_ART_R_11	7	15
12	MZ_ART_F_5	MZ_ART_R_17	4	1
12	MZ_ART_F_4	MZ_ART_R_4	4	1
13	MZ_ART_F_3	MZ_ART_R_5	4	1
13	MZ_ART_F_5	MZ_ART_R_18	4	1
14	MZ_ART_F_3	MZ_ART_R_6	4	5
14	MZ_ART_F_4	MZ_ART_R_6	4	1
16	MZ_ART_F_3	MZ_ART_R_7	4	15

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16	MZ_ART_F_4	MZ_ART_R_7	4	5
17	MZ_ART_F_3	MZ_ART_R_8	4	0
17	MZ_ART_F_4	MZ_ART_R_8	4	0
20	MZ_ART_F_3	MZ_ART_R_10	4	0
20	MZ_ART_F_4	MZ_ART_R_10	4	0
21	MZ_ART_F_3	MZ_ART_R_11	4	0
21	MZ_ART_F_4	MZ_ART_R_11	4	0
[illegible]	MZ_ART_F_5	MZ_ART_R_1	4	5
[illegible]	MZ_ART_F_6	MZ_ART_R_1	4	5
[illegible]	MZ_ART_F_5	MZ_ART_R_2	4	1
[illegible]	MZ_ART_F_6	MZ_ART_R_2	4	1
[illegible]	MZ_ART_F_5	MZ_ART_R_3	4	1
[illegible]	MZ_ART_F_6	MZ_ART_R_3	4	1
[illegible]	MZ_ART_F_6	MZ_ART_R_4	4	5
[illegible]	MZ_ART_F_5	MZ_ART_R_15	4	5
[illegible]	MZ_ART_F_5	MZ_ART_R_5	4	5
26	MZ_ART_F_6	MZ_ART_R_5	4	5
27	MZ_ART_F_5	MZ_ART_R_6	4	0
27	MZ_ART_F_6	MZ_ART_R_6	4	0
28	MZ_ART_F_5	MZ_ART_R_7	4	1
28	MZ_ART_F_6	MZ_ART_R_7	4	1
29	MZ_ART_F_5	MZ_ART_R_8	4	5
29	MZ_ART_F_6	MZ_ART_R_8	4	5
30	MZ_ART_F_5	MZ_ART_R_9	4	1
30	MZ_ART_F_6	MZ_ART_R_9	4	1
31	MZ_ART_F_5	MZ_ART_R_10	4	5
31	MZ_ART_F_6	MZ_ART_R_10	4	5
32	MZ_ART_F_5	MZ_ART_R_11	4	5
32	MZ_ART_F_6	MZ_ART_R_11	4	1
33	MZ_ART_F_7	MZ_ART_R_1	4	5
33	MZ_ART_F_8	MZ_ART_R_1	4	15
34	MZ_ART_F_7	MZ_ART_R_2	4	0
34	MZ_ART_F_8	MZ_ART_R_2	4	0
35	MZ_ART_F_7	MZ_ART_R_3	4	5
35	MZ_ART_F_5	MZ_ART_R_13	4	1
36	MZ_ART_F_7	MZ_ART_R_4	4	5
36	MZ_ART_F_8	MZ_ART_R_4	4	1
37	MZ_ART_F_7	MZ_ART_R_5	4	5
37	MZ_ART_F_8	MZ_ART_R_5	4	1
38	MZ_ART_F_7	MZ_ART_R_6	4	15

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38	MZ_ART_F_8	MZ_ART_R_6	4	1
39	MZ_ART_F_7	MZ_ART_R_7	4	15
39	MZ_ART_F_8	MZ_ART_R_7	4	5
40	MZ_ART_F_7	MZ_ART_R_8	4	5
40	MZ_ART_F_8	MZ_ART_R_8	4	15
41	MZ_ART_F_7	MZ_ART_R_9	4	0
41	MZ_ART_F_8	MZ_ART_R_9	4	0
42	MZ_ART_F_7	MZ_ART_R_10	4	1
42	MZ_ART_F_8	MZ_ART_R_10	4	5
43	MZ_ART_F_7	MZ_ART_R_11	4	1
43	MZ_ART_F_8	MZ_ART_R_11	4	5
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44	MZ_ART_F_4	MZ_ART_R_20	4	0
45	MZ_ART_F_2	MZ_ART_R_16	4	5
45	MZ_ART_F_4	MZ_ART_R_16	4	5
46	MZ_ART_F_2	MZ_ART_R_17	4	0
46	MZ_ART_F_4	MZ_ART_R_17	4	0
47	MZ_ART_F_2	MZ_ART_R_18	4	0
47	MZ_ART_F_4	MZ_ART_R_18	4	0
31r	MZ_ART_F_1	MZ_ART_R_15	4	5
31r	MZ_ART_F_3	MZ_ART_R_15	4	1
32r	MZ_ART_F_1	MZ_ART_R_16	4	5
32r	MZ_ART_F_3	MZ_ART_R_16	4	1
33r	MZ_ART_F_1	MZ_ART_R_17	4	5
33r	MZ_ART_F_3	MZ_ART_R_17	4	1
37r	MZ_ART_F_1	MZ_ART_R_18	4	5
37r	MZ_ART_F_3	MZ_ART_R_18	4	1
40r	MZ_ART_F_1	MZ_ART_R_19	4	1
40r	MZ_ART_F_3	MZ_ART_R_19	4	1

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APPENDIX 3: SUMMARY OF NUMBER OF PELLETS CONTAINING INSECT PREY ITEMS (SPECIES)

Order	Family	Genus	Species	*N. thebaica* (colony)	*C. pumilus* (N=2 pellets, 1 bat)	*M. midas* (colony)	*S. dinganii* (N=4 pellets, 2 bats)	*P. hesperidus* (N=4 pellets, 3 bats)	*H. caffer* (N=1 pellet, 1 bat)
Blattaria	Blaberidae	*Pycnoscelus*		1		1			
Blattaria	Other	*Other*	Other	3	1	6	1	1	
Coleoptera	Dytiscidae	*Cybister*	*tripunctatus*	1		10			
Coleoptera	Other	*Other*	Other	1	1	7	2	2	1
Diptera	Chloropidae					2		2	
Diptera	Culicidae	*Culex*	*quinquefasciatus*					1	
Diptera	Sciaridae							1	
Hemiptera	Other	*Other*	Other		1	3	1		
Hemiptera	Pentatomidae	*Macrorhaphis*	*acuta*	1	1	4	1		
Hemiptera	Pentatomidae	*Nezara*	*Nezara_viridula*	1	1	6	2	2	
Lepidoptera	Castniidae	*Synemon*	*austera*	1		1			
Lepidoptera	Crambidae	*Talanga*	*sexpunctalis*			1			
Lepidoptera	Elachistidae	*Antaeotricha*			1	1			
Lepidoptera	Elachistidae	*elachJanzen01*	*Janzen109*			1			
Lepidoptera	*Erebidae	*Araeopteron*				8		1	
Lepidoptera	*Erebidae	*Dyops*	*subdifferens*			1			
Lepidoptera	*Erebidae	*Eublemma*				1			
Lepidoptera	*Erebidae	*Peridrome*	*orbicularis*			1			
Lepidoptera	*Erebidae	*Spargaloma*	*sexpunctata*			1			
Lepidoptera	*Erebidae	*Toxocampinae*	*Tathorhynchus*	2	1	18	1	1	
Lepidoptera	Euteliidae	*Eutelia*	*polychordaUB01*	1		8		1	
Lepidoptera	Euteliidae	*Lophoptera*				2			

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Lepidoptera	Euteliidae	*Phlegetonia*	*callichroma*			1		1			
Lepidoptera	*Geometridae	*Acanthovalva*	*inconspicuaria*	2		14					
Lepidoptera	*Geometridae	*Achrosis*					1				
Lepidoptera	*Geometridae	*Achrosis*	*pyrrhularia*			1					
Lepidoptera	*Geometridae	*Aethalura*	*punctulata*			9					
Lepidoptera	*Geometridae	*Cabera*	*AH02SA*			1					
Lepidoptera	*Geometridae	*Chiasmia*	*interrupta*			1		1			
Lepidoptera	*Geometridae	*Ennominae*	Other			14		1			
Lepidoptera	*Geometridae	*Eupithecia*				2					
Lepidoptera	*Geometridae	*Isturgia*	*arizeloides*			7					
Lepidoptera	*Geometridae	*Isturgia*	*roraria*	2	1	22	2	2			
Lepidoptera	*Geometridae	*Isturgia*	*pulinda*			3					
Lepidoptera	*Geometridae	*Scopula*	*rufisalsa*	1							
Lepidoptera	*Geometridae	*Scopula*	*atricapilla*			2					
Lepidoptera	Hyblaeidae	*Hyblaea*	*puera*	1		3					
Lepidoptera	Lasiocampidae	*Gonometa*	*postica*			3					
Lepidoptera	Lasiocampidae	*Sena*		1	1	5	2	2	1		
Lepidoptera	Lasiocampidae	*Sena*	*prompta*	1	1	3	1	1			
Lepidoptera	Limacodidae					1	2	2			
Lepidoptera	Mimallonidae	*Mimallo*	*amiliaDHJ01*						1		
Lepidoptera	*Noctuidae	*Xenopseustis*				2		1	1		
Lepidoptera	*Noctuidae	*Acronicta*	*psi*			2					
Lepidoptera	*Noctuidae	*Adisura*	*marginalis*			3					
Lepidoptera	*Noctuidae	*Athetis*	*ignava*			4		1			
Lepidoptera	*Noctuidae	*Bastilla*	*torrida*			2					
Lepidoptera	*Noctuidae	*Bryophila*	*AH01Pe*			2					
Lepidoptera	*Noctuidae	*Chasmina*	*lispodes*			5					
Lepidoptera	*Noctuidae	*Chasmina*				1					
Lepidoptera	*Noctuidae	*Condica*	*parista*			1					
Lepidoptera	*Noctuidae	*Copitarsia*				5		1			

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Lepidoptera	*Noctuidae	*Cortyta*	*canescens*		1	1	1	1	
Lepidoptera	*Noctuidae	*Enmonodia*	*capensis*	1	1	1		1	
Lepidoptera	*Noctuidae	*Grammodes*	*stolida*			1			
Lepidoptera	*Noctuidae	*Harrisimemna*	*trisignata*			6			
Lepidoptera	*Noctuidae	*Heterochroma*	*BioLep767*			1			
Lepidoptera	*Noctuidae	*Hypotacha*				8			
Lepidoptera	*Noctuidae	*Leucania*	*imperfecta*		1				
Lepidoptera	*Noctuidae	*Leucania*	*BioLep10*	1		8			
Lepidoptera	*Noctuidae	*Leucania*	*BioLep162*			2			
Lepidoptera	*Noctuidae	*Leucania*	*multilinea*			1			
Lepidoptera	*Noctuidae	*Leucania*	*Poole05*			2			
Lepidoptera	*Noctuidae	*Leucania*	*porphyrodes*			1			
Lepidoptera	*Noctuidae	*Mythimna*	*consanguis*			4			
Lepidoptera	*Noctuidae	*Mythimna*	*convecta*			3			
Lepidoptera	*Noctuidae	*Mythimna*	*impura*			5		1	
Lepidoptera	*Noctuidae	*Mythimna*	*oxygala*			1		1	
Lepidoptera	*Noctuidae	*Mythimna*	*nigrisparsa*			1			
Lepidoptera	*Noctuidae	*Mythimna*	*obsoleta*			3			
Lepidoptera	*Noctuidae	*Mythimna*				4			
Lepidoptera	*Noctuidae	*Niphonyx*	*segregata*			1			
Lepidoptera	*Noctuidae	*Hadenini*				1			
Lepidoptera	*Noctuidae	*Ophiusa*	*tirhaca*			2			
Lepidoptera	*Noctuidae	*Pandesma*	*robusta*	2		20	1	2	
Lepidoptera	*Noctuidae	*Paratrachea*	*laches*			1			
Lepidoptera	*Noctuidae	*Pericyma*	*atrifusa*	1	1	14	1	2	1
Lepidoptera	*Noctuidae	*Argyrogrammatini*		1			1		
Lepidoptera	*Noctuidae	*Polia*	*piniae*			2			
Lepidoptera	*Noctuidae	*Rhesala*	*moestalis*		1	2			
Lepidoptera	*Noctuidae	*Spodoptera*	*exigua*		1	7	1	1	
Lepidoptera	*Noctuidae	*Thysanoplusia*	*indicator*	1					

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Lepidoptera	*Noctuidae	*Tycomarptes*		2		5	1	1	1
Lepidoptera	*Noctuidae	*Xanthodes*	*malvae*			1			
Lepidoptera	*Noctuidae	Other	Other	4	1	20	2	3	1
Lepidoptera	Nolidae	*Garella*	*nilotica*	1		2			
Lepidoptera	Nolidae	*Maurilia*	*arcuata*	1	1	1	2	1	1
Lepidoptera	Nolidae	*Meganola*	*dentata*			1			
Lepidoptera	*Notodontidae	*Disphragis*	*baracoanaDHJ02*			1			
Lepidoptera	*Notodontidae	*Pseudosomera*	*noctuiformis*			1			
Lepidoptera	Other	Other	Other			19	1	1	
Lepidoptera	Pterophoridae	*Stenoptilia*			1	1			
Lepidoptera	*Pyralidae	*Phycitinae*	Other	1		4			
Lepidoptera	Saturniidae	*Copaxa*	*lavendera*			1			
Lepidoptera	Saturniidae	*Pseudautomeris*	*irene*			1		1	
Lepidoptera	Scythrididae	*Arotrura*	*JFL201*	1				1	
Lepidoptera	*Sphingidae	*Agrius*	*convolvuli*			5		1	
Lepidoptera	Tortricidae	*Cochylidia*	*implicitana*			2			
Lepidoptera	Tortricidae	*Platphalonidia*	*felix*			3			
Neuroptera	Other	Other	Other			7			
Orthoptera	Other	Other	Other		1	1	1	1	
Orthoptera	Tettigoniidae	*Neoconocephalus*	*spiza*				1		
Orthoptera	Tettigoniidae	Tettigoniinae	Other	2	1	5	2	2	

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The insect prey items were determined by Next Generation Sequencing of the CO1 gene in faecal pellets of six genera of bats occurring around macadamia orchards in Levubu, South Africa. Families of moths known to have hearing organs sensitive to bat echolocation based on literature (Sierra & Arlettaz 1997; Zha *et al.* 2009) are indicated with asterisks.

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Bats eat pest green vegetable stink bugs (*Nezara viridula*): Diet analyses of seven insectivorous species of bats roosting and foraging in macadamia orchards

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ABSTRACT

A growing body of literature has documented the economically significant impact of bats as predators of agricultural pest insects. This has not yet been established in macadamia agro-ecosystems where stink bugs (Hemiptera: Pentatomidae) are the major pests. The present study examined faecal pellets of seven species of bats collected from macadamia orchards in the Levubu Valley, Limpopo Province, South Africa, using both conventional microscopic as well as second generation sequencing analyses (faecal pellets from three bat species were studied by both methods). Microscopic analysis of faecal pellets and culled prey remains collected from night and day roosts and captured individuals of slit-faced bats (*Nycteris thebaica*), Angolan free-tailed bats (*Mops condylurus*), African pipistrelles (*Pipistrellus hesperidus*) and yellow house bats (*Scotophilus dinganii*) revealed important (20-50%) proportions of bugs (Order Hemiptera), higher than recorded in previous studies conducted in natural habitats. But this method was unable to provide further taxonomic resolution concerning the families or species of bugs present and whether these included the pest green vegetable stink bug (*Nezara viridula* - also known in the USA as the southern green stink bug). Preliminary results from a molecular approach, using second generation sequencing of a 157 bp fragment of the COI-barcoding region, revealed that faecal pellets of five species of bats foraging and roosting in macadamia plantations contained DNA from the pest green vegetable stink bug: slit-faced bats (*Nycteris thebaica*), Mops free-tailed bats (*Mops midas*), little free-tailed bats (*Chaerephon pumilus*), African pipistrelles (*Pipistrellus hesperidus*) and yellow house bats (*Scotophilus dinganii*). No stink bug DNA was detected in pellets of a sixth species, Sundevall's leaf-nosed bat (*Hipposideros caffer*). Based on a total of 37 faecal pellets analysed for all bat species combined, about one third (32%) of the pellets contained DNA from green vegetable stink bugs. A similar proportion of the pellets (35%) contained DNA from unidentified insects from the order Hemiptera. These data provide unequivocal evidence for predation by bats on green vegetable stink bugs, but the economic importance remains to be established although it is likely to be high.

UITTREKSEL

Meer inligting oor die ekonomies-belangrike rol wat vlermuise as predatore speel in die bekamping van insekplae in die landbou word toenemend gepubliseer. Die rol van vlermuise in die bestryding van stinkbesies (Hemiptera: Pentatomidae) as dié belangrikste plaaginsek in makadamia-boorde, is egter nog nie omskryf nie. Die onderhawige studie bevat 'n ontleding van vlermuismis van sewe vlermuisspesies wat in makadamia-boorde in die Levubu-vallei, Limpopo-provinsie, Suid-Afrika, voorkom. Ontledings is met behulp van tradisionele mikroskopiese analises, asook tweede-generasie DNS-ontleding, op die mis van drie

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spesies gedoen. Mikroskoop-analises van vlermuismis en ander prooi-reste van *Nycteris thebiaca*, *Mops condylurus*, *Pipistrellus hesperidus* en *Scotophilus dinganii* wat by nagvoedingstasies en slaapskuilings (roosts) versamel is, asook die mis van individue wat in nette gevang is, het aan die lig gebring dat 'n belangrike deel (20-50%) van die vlermuise se dieet, insekte van die familie Hemiptera bevat. Hierdie syfers is hoër is as dié wat in vorige misontledings verkry is wat in natuurlike habitatte uitgevoer is. Hierdie metode kon egter nie meer taksonomiese besonderhede verskaf oor die families of spesies van Hemiptera teenwoordig nie, en of dit die groen stinkbesie (*Nezara viridula*) insluit nie. Die voorlopige resultate van tweede-generasie DNS-ontledings van die COI-geen het egter getoon dat die vlermuismis van vyf van die vlermuisspesies wat in makadamia-boorde vir prooi jag, DNS van die groen stinkbesie bevat; hierdie vyf spesies is *Nycteris thebiaca*, *Mops midas*, *Chaerephon pumilus*, *Pipistrellus hesperidus* en *Scotophilus dinganii*. Geen sodanige DNS van stinkbesies kon in die mis van 'n sesde spesie, *Hipposideros caffer*, gevind word nie. Uit al die spesies se totale ontledings van 37 mismonsters het ongeveer 'n derde (32%) DNS van die groen stinkbesiespesie bevat. 'n Soortgelyke hoeveelheid (35%) van die mismonsters het DNS van ongeïdentifiseerde Hemiptera bevat. Hierdie inligting bewys onteenseglik dat stinkbesies 'n beduidende hoeveelheid van insekvretende vlermuise se dieet uitmaak; die ekonomiese impak moet egter nog bepaal word, hoewel dit waarskynlik ook aansienlik groot is.

INTRODUCTION

Insect pests cause significant damage to agricultural production worldwide. Bat predation of pest insects has been shown to have a significant economic benefit to farmers (Cleveland *et al.*, 2006; Boyles *et al.*, 2011; Kunz *et al.*, 2011). In macadamia plantations in South Africa, stink bugs (Hemiptera: Pentatomidae) are the major pests of macadamias (Ironside, 1973; Jones & Caprio, 1992; Jones *et al.*, 1992; Jones & Caprio, 1994; Vincent *et al.*, 2001; Schoeman & Mohlala, 2012).

Very few African studies have examined the economic impact of bats eating agricultural pest insects. In sugarcane plantations in Swaziland, Noer *et al.* (2012) showed that two molossid bat species (*Chaerephon pumilus* and *Mops condylurus*) roosting near sugarcane plantations, selectively forage over these fields, while Bohmann *et al.* (2010 & 2011) showed that these bats feed on insect species such as boring moths (*Eldana saccharina* and *Mythimna phaea*) and stink bugs (Hemiptera, Pentatomidae).

There is an urgent need to demonstrate the economic value of bats to agriculture to promote awareness of the need for their conservation. Increasing local bat populations (*e.g.* by erecting bat houses) may prove to be an effective and less environmentally destructive means of keeping insect pest numbers down, than simply increasing chemical pesticide use which may ultimately prove counterproductive if populations of beneficial top insects predators like insectivorous spiders, bats and birds decline through poisoning (Wickramasinge *et al.*, 2003; Pocock & Jennings, 2008).

Previous studies (Taylor *et al.*, 2011, 2012a, b) demonstrated that a variety of local bat species forage in macadamia orchards and that seasonal variation of foraging activity of bats in macadamia orchards corresponds with the times of the year when major infestations of pest insects are known to occur in local macadamia orchards, *e.g.* November to January in macadamia nut borers, *Thaumatotibia batracopa*, and late austral summer to autumn in stink bugs.

The objective of this study was to establish baseline data on the diet of different bat species resident in macadamia agro-ecosystems, with a view to identifying possible insect pest families, genera and species. In particular, we were interested to establish whether bats consumed the primary pest of macadamias, green vegetable stink bugs (*Nezara viridula*) or other pest pentatomid species (such as *Bathycoelia natalicola*). To meet this objective, we examined the diet of seven bat species with very different foraging strategies, including clutter-feeders (the slit-faced bat, *Nycteris thebaica*, and Sundevall's leaf-nosed bat, *Hipposideros caffer*), clutter-edge foragers (the African pipistrelle, *Pipistrellus hesperidus*, and the yellow house bat, *Scotophilus dinganii*) and open-air foragers (the Angolan free-tailed bat, *Mops condylurus*, Mops midas bat, *Mops midas*, and the little free-tailed bat, *Chaerephon pumilus*) (see Monadjem *et al.*, 2010a for a summary of bat foraging strategies). We collected faecal pellets from known roosts of these bats in the area, as well as from captured individuals flying and presumably foraging in or near macadamia orchards. We used both conventional microscopic methods (Whitaker, 1988; Whitaker, McCracken & Siemers, 2009) as well as a second generation sequencing approach (modified from Bohmann *et al.*, 2011).

Although we would predict that clutter and clutter-edge bats would be more likely than aerial feeding bats to prey on predominantly slow-flying stink bugs which usually fly close to vegetation, Bohmann *et al.* (2011) recorded DNA of stink bugs (Pentatomidae) in eight out of 89 pellets (14%) analysed from aerial-feeding free-tailed bats (*Mops condylurus* and *Chaerephon pumilus*) in a sugarcane-dominated landscape in Swaziland. We thus predict that most of the bat species sampled would prey on locally abundant pest stink bug (and other) pest species, especially during the peak stink bug outbreak seasons of late summer and autumn (Schoeman & Mohlala, 2012; Taylor *et al.*, 2012a).

MATERIALS AND METHODS

Study area

The study area is located in the southern foothills of the Soutpansberg range, 20 km east of the town of Makhado (previously, Louis Trichardt), encompassing the Levuvhu Valley subtropical fruit-growing area in the south, which is dominated by extensive monocultures of macadamias, pecan nuts, avocados, bananas and pine and gum plantations (Fig. 1). It is bordered to the north by mountains covered predominantly by dense thickets classified by Mucina and Rutherford (1996) as "Soutpansberg Mountain Bushveld", as well as by extensive commercial plantations of gums and pines. Annual rainfall in the study area was around 930 mm in 2010 and 960 mm in 2011 and fell mostly between November and

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April. Daily maximum temperatures frequently exceed 35°C in summer (October to March) but seldom exceed 40°C and minimum daily summer temperature seldom fall below 15°C, whereas winters are much colder, with minimum daily temperatures dropping to just over 0°C on a few days in June and July and maximum daily temperatures reaching 25°C (Taylor *et al.*, 2012a). The study area is known to be a species-rich hotspot for bats (Taylor *et al.*, 2012a, c).

Collection of faecal pellets

In all cases where faecal pellets were obtained from day or night roosts (for three species), they were collected onto 1 x 2 m boards covered in clingfilm placed under the position of roosting bats approximately one week prior to collection. Pellets of the remaining four species were obtained from captured individual bats, that were held individually in cloth bags until they yielded sufficient numbers of pellets. In the case of

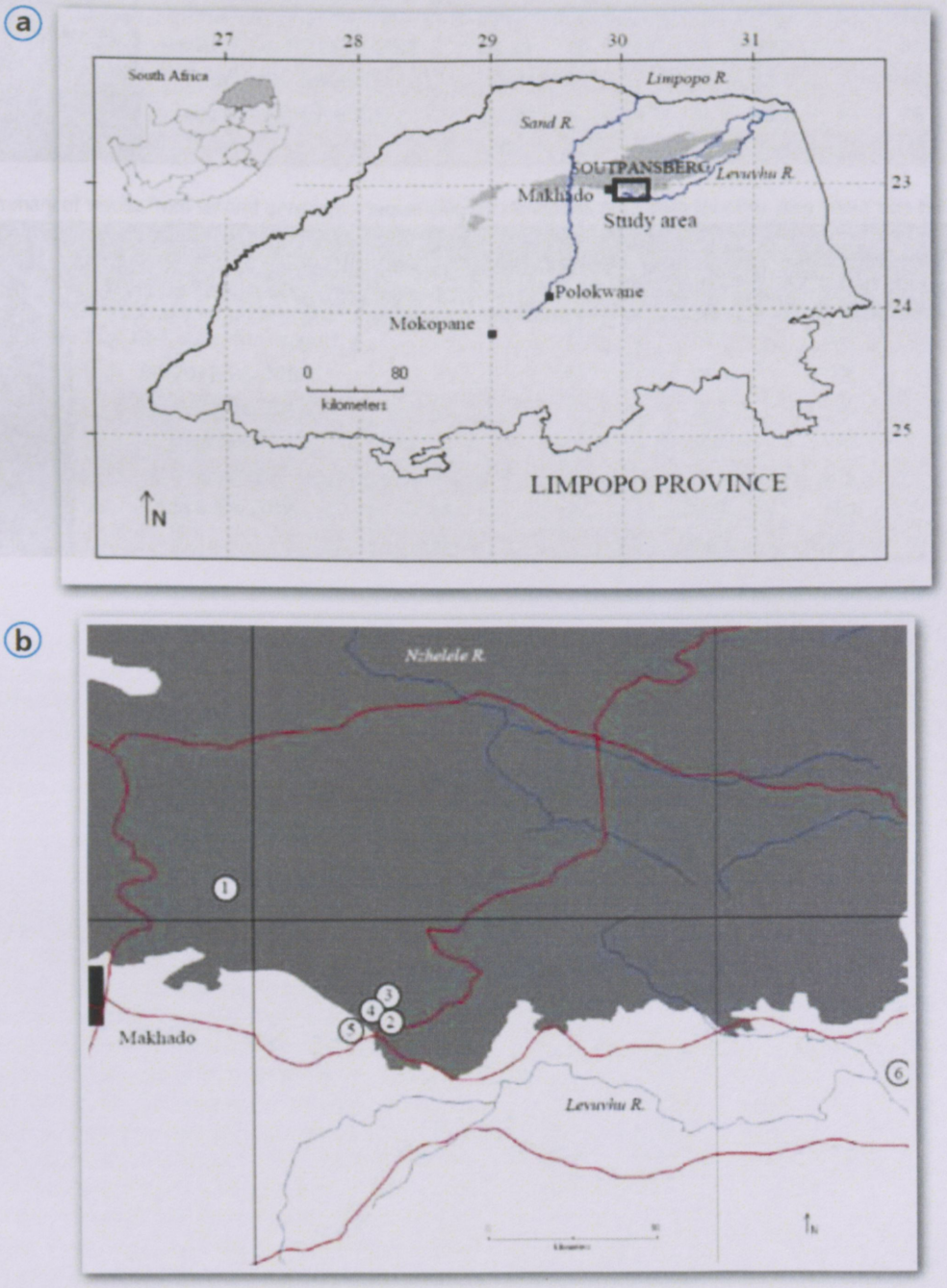


Figure 1. (a) Map of study area showing location of study area in South Africa's Limpopo Province and (b) detailed location of pellet collection sites within the study area from Farm Vlakfontein position 1, Farm Welgevonden, positions 2, 3, 4 and 5, and Farm Laatsgevonden position 6. Grey shading indicates the extent of vegetation types associated with the Soutpansberg Range.

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Pipistrellus hesperidus (n=2) and *Hipposideros caffer* (n=1), these were from harp-trapped individuals, whereas in *Scotophilus dinganii* (n=3) and *Chaerephon pumilus* (n=1), these were from individuals roosting in an attic, which periodically flew accidentally into the house. Collection sites were primarily from positions 3 (day-roost of *M. condylurus*), 4 (day roost of both *M. midas* and *M. condylurus*), 2 (day roost of *S. dinganii* and *C. pumilus*) and 5 (harp trap site for collection

Table 1. Summary of faecal pellet collections used for microscopic dissection for identification of arthropod prey items in the diet of four species of bats and summary of proportions (by volume) of unidentified Hemiptera.

Species	Sample months	No. pellets analysed	No. prey orders	Hemiptera % volume	Hemiptera % occurrence
N. thebaica	July, Aug, Oct, Nov 2010	20, 4	5	19.5	25.0
S. dinganii	Feb 2011, Feb 2012, Oct 2012	24, 24	5	30.3	91.7
P. hesperidus	Sept 2011, Jan 2012	18, 4	4	50.0	75.0
M. condylurus	Apr, Nov 2010, Feb 2012	30, 30	4	38.0	76.7
Species combined		92	8	34.4	79

Table 2. Summary of results from second generation sequencing for Hemiptera prey (data for other prey insect taxa not shown).

				% occurrence (no. of pellets) of Hemiptera		
Species	Sample months	No. pellets analysed	No. prey orders	*N. viridula*	*M. acuta*	Hemiptera (not identified)
N. thebaica	May 2012, Aug 2010, Aug 2012	6	5	16.7	16.7	16.7
S. dinganii	Feb 2011, Feb 2012	2	5	100	50	100
P. hesperidus	Aug 2011, Jan 2012	4	6	66.7	0	66.7
C. pumilus	Apr 2012	1	5	100	100	100
H. caffer	Aug 2012	1	2	0	0	0
M. midas	Aug & Nov 2012	24	7	25.0	16.7	29.2
Species combined		37		32.4	18.9	35.14

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of *P. hesperidus* and *H. caffer* individuals) of the farm Welgevonden, with one site located approximately 10 km to the northeast (the farm Vlakfontein, a day-roost of *M. midas*) and another 30 km to the east (the farm Laatsgevonden, a night-roost of *N. thebaica*) (Fig. 1). Pellets to be analysed microscopically were kept in ziplock bags at 4°C prior to analysis, while pellets to be analysed molecularly were stored in 90% ethanol at 4°C.

Microscopic dissection of faecal pellets

Wherever possible, a minimum of five pellets were analysed for each sample as described by Whitaker (1988) and Whitaker, McCracken & Siemers (2009). Each pellet was teased apart after adding 70% ethanol and the arthropod exoskeleton fragments were identified to order (or family if possible) using the standard key in Whitaker (1988), Whitaker, McCracken & Siemers (2009), drawings in Scholtz & Holm (1985) and a reference collection of arthropods (insects and spiders) trapped with malaise traps in macadamia orchards during two nights in December 2010 and January 2011 at Farm Welgevonden (Fig. 1, position 2). For each pellet, the percentage by volume of the different prey orders was estimated.

Identification of prey-DNA in faecal pellets

A detailed account of the molecular laboratory procedures followed is beyond the scope of this article but will be described elsewhere (Taylor *et al.*, in preparation; for similar approaches, see also Binladen *et al.*, 2007; Meyer & Kircher, 2010; Bohmann *et al.*, 2010, 2011; & Zeale *et al.*, 2011). The main steps are briefly summarised below.

Second generation sequencing of prey-insect DNA in faecal pellets

In summary, insect DNA was extracted from 44 unique bat faecal pellets. In order to identify insect-prey in the bat droppings, amplification and second generation sequencing of ca. 157 bp insect-COI fragments (Zeale *et al.*, 2011) were performed on the faecal extracts following a modification of the methods described in Bohmann *et al.*, 2011. The assay described here was customised for Illumina MiSeq sequencing as opposed to GS FLX sequencing used by Bohmann *et al.*, 2011.

Sequencing of pest insects

Already detached legs from collected specimens were taken from each of 11 insect (Hemiptera) species collected from local macadamia plantations by JNS and identified by Dr M Stiller of the Agricultural Research Council. This list included three known macadamia pest species and an additional eight species which are not known to be pests. The pest species included *Nezara viridula* (Pentatomidae; green vegetable stink bug), *Pseudotheraptus wayi* (Coreidae; coconut bug) and *Bathycoelia rodhaini* (Pentatomidae; yellow-spotted stink bug). The non-pest species included *Dalsira costalis* (Pentatomidae; stink bug), *Caurarufi ventris* (Pentatomidae; stink bug), *Coridius nubilis* (Pentatomidae; stink bug), *Anoplecnemis curvipes* (Coreidae; giant coreid bug), *Leptoglossus australis* (Coreidae; leaf-footed plant bug), *Veterna* sp. (Pentatomidae; grass stink bugs) and *Dysdercus nigrofasciata* (Pyrrhocoridae; cotton stainer, a major pest of cotton). DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Extracts were amplified with Folmer primers (LCO1490, HCO2198) (Folmer *et al.*, 1994) amplifying a 648 bp COI-fragment. Purification and Sanger sequencing of the products (both directions) were undertaken by the commercial facility offered by Macrogen (Seoul, South Korea).

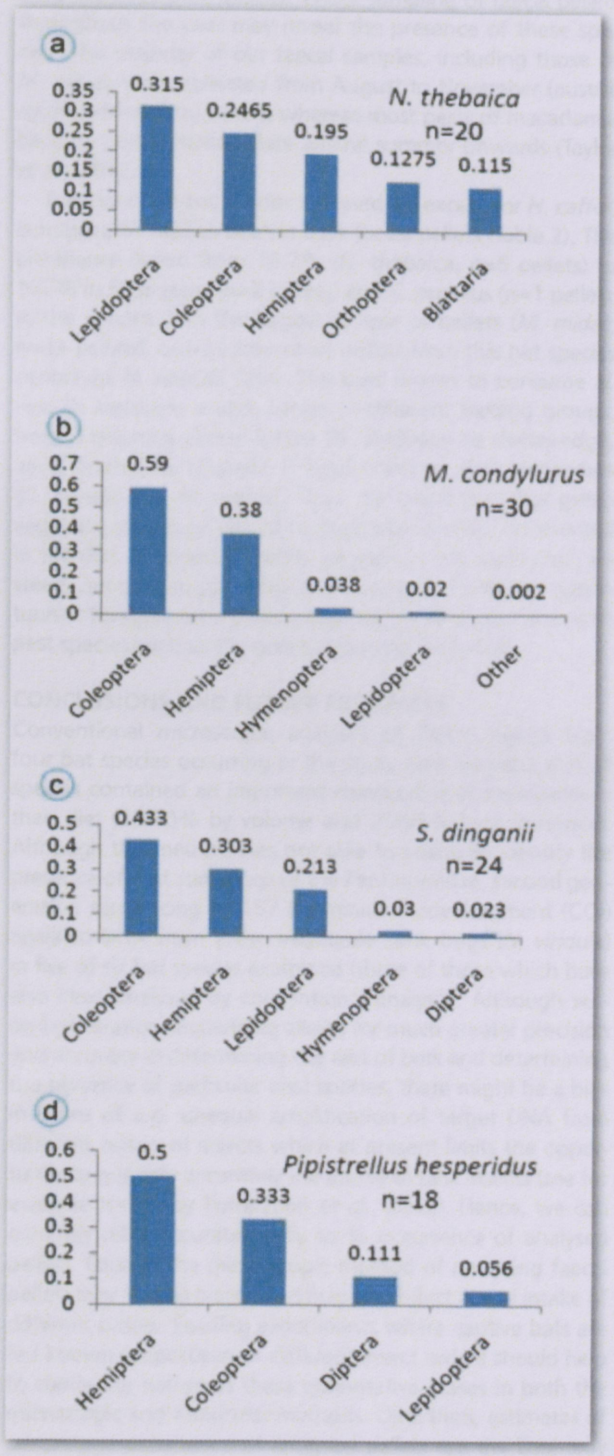


Figure 2. Bar charts showing percentage volume of different orders of prey insects in the diet of four bat species analysed using microscopic dissection of faecal pellets.

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Sequence analyses

Full details are provided elsewhere (Taylor *et al.*, in preparation). COI barcode sequences from different taxonomic levels (Actinopterygii, Arachnida, Branchiopoda, Chilopoda, Insecta, Isopoda, Ostracoda and Sarcopterygii) were downloaded from the BOLD v3 (Ratnasingham & Hebert, 2007) Public Data Portal to create a database and its corresponding taxonomy map. Furthermore, the sequences of the eleven hemipteran insects that were sequenced in this study were added to the database. Species identification was made with QIIME v1.7.0 (Caporaso *et al.*, 2010).

RESULTS AND DISCUSSION

Dietary analysis by microscopic analysis of faecal pellets

Fig. 2 summarises proportions (by volume) of different orders of insects in the diet of four species of bats based on microscopic examination of 92 faecal pellets collected opportunistically between 2010 and 2012 (Table 1). All species contained an important component of unidentified Hemiptera in their diet (means of 20-50% by volume; 25-92% by occurrence) (Fig. 2, Table 1). Four to five different orders of insects (prey items) could be determined per species (eight in total). Given that this method relies on the identification of prey remains, which have been through the digestive system, further taxonomic resolution was not possible, *e.g.* we were unable to determine the presence of stink bugs (Pentatomidae or Coreidae) in general, or of individual species such as the green vegetable stink bugs (*N. viridula*).

The diet of two of these species (*N. thebaica* and *M. condylurus*) were reported in detail previously by Taylor *et al.* (2011) and Taylor *et al.* (2012c), who showed that the proportion of Hemiptera was higher than reported from previous studies conducted in natural areas. Likewise, the new data presented here for *S. dinganii* and *P. hesperidus* indicate relatively high proportions of Hemiptera (30.3 and 50.5% respectively), compared with previous studies. Based on five previous studies of *S. dinganii* conducted in natural habitats in South Africa and Zimbabwe, the proportion of Hemiptera was much lower, varying from 0 to 3.7% (Aldridge & Rautenbach, 1987; Fenton *et al.*, 1998; Schoeman, 2006; Jacobs & Barclay, 2009; Schoeman & Jacobs, 2011; Tshitande, 2011). In the above-mentioned studies, Coleoptera dominated in the diet of *S. dinganii* (42-100%), as found also in our study (43.4%). In the case of *P. hesperidus* it is known to be a bug-specialist and previous studies have shown Hemiptera to be an important component in the diet (33-40%) (Schoeman, 2006; Schoeman & Jacobs, 2011; Tshitande, 2011), only slightly lower than found in our study (50%).

Dietary analysis by second generation sequencing

Of the 44 pellets initially extracted, 37 were successfully amplified with insect generic minibarcode primers (Zeale *et al.*, 2011) and sequenced. Here, given the relevance to biological control of stink bugs, we report only on the Hemiptera component of the diet. More detailed and broader taxonomic analysis of the diet of the six species investigated will be reported elsewhere (Taylor *et al.*, in preparation). The molecular analyses identified the green vegetable stink bug (*N. viridula*) and the predatory pentatomid, *Macrorhaphis acuta*, among the Hemiptera sequences. Combining data for all species, 32% of pellets contained *N. viridula*, 19% contained *M. acuta* and 35% contained unidentified Hemiptera (Table 2). Apart from *N. viridula*, no DNA matches were obtained for the other 10 hemipteran species sequenced by this study (see Methods) and which are known to occur on macadamia farms. Very little is known about the life cycle of these bug species, which included three pest species. Wider sampling of faecal pellets throughout the year may reveal the presence of these species. The majority of our faecal samples, including those of *M. midas*, were collected from August to November (austral spring and early summer), whereas most pests of macadamia become prevalent from late austral summer onwards (Taylor *et al.*, 2012b).

Five out of six bat species analysed (all except for *H. caffer*) contained *N. viridula* DNA in their faecal pellets (Table 2). The prevalence varied from 16.7% (*N. thebaica*, n=6 pellets) to 100% in *S. dinganii* (n=2 pellets) and *C. pumilus* (n=1 pellet). In the species with the largest sample of pellets (*M. midas*; n=24 pellets), one quarter of all pellets from this bat species contained *N. viridula* DNA. The bats shown to consume *N. viridula* represent a wide range of different feeding groups, from a gleaning clutter-feeder (*N. thebaica*) to clutter-edge, aerial feeders (*S. dinganii*, *P. hesperidus*) to open-air feeders (*C. pumilis* and *M. midas*). Thus, our prediction that green vegetable stink bugs would be disproportionately represented in the diet of gleaning clutter foragers is not supported; instead, our data suggest that a wide range of bats are opportunistic foragers taking advantage of the local abundance of pest species such as the green vegetable stink bug.

CONCLUSIONS AND FUTURE PROSPECTS

Conventional microscopic analyses of faecal pellets from four bat species occurring in the study area revealed that all species contained an important component of Hemiptera in their diet (20-50% by volume and 25-92% by occurrence). Although this method was not able to positively identify the presence of pest stink bugs of the Pentatomidae, second generation sequencing of 157 bp mini-barcode fragment (COI) revealed DNA from green vegetable stink bugs (*N. viridula*) in five of six bat species examined (three of these which have also been analysed by conventional analysis). Although second generation sequencing allows for much greater precision and accuracy in determining the diet of bats and determining the presence of particular pest species, there might be a bias in terms of *e.g.* unequal amplification of target DNA from different orders of insects which at present limits the opportunity to quantify accurately the intake of pest insects (see for example review by Pompanon *et al.*, 2012). Hence, we can currently refer accurately only to % occurrence of analysed pellets. Equally, the microscropic method of analysing faecal pellets may also be biased and may not reflect actual intake of different orders. Feeding experiments where captive bats are fed known proportions of different insect orders should help to clarify the nature of these quantitative biases in both the microscopic and molecular methods. Until then, estimates of percentage occurrence of analysed pellets are the best way to present the results. Indeed, when we consider percentage occurrence for the microscopic results, we found similar occurrence of Hemiptera to the values obtained for unidentified

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Hemiptera and for *N. viridula* in the molecular results, when we compare results for the same bat species (Tables 1 and 2). Thus, microscopic and DNA methods respectively showed percentage occurrence of Hemiptera or *N. viridula* as 25% and 17% for *N. thebaica*, 75% and 67% for *P. hesperidus* and 92% and 100% for *S. dinganii*.

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