Phylogeny of Emperor moths and phylogeography of *Gonimbrasia belina* in Southern Africa

by

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Declaration

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Part of this thesis was included in a conference proceedings paper, of which I was the principal responsible. Part of this thesis also was included in a subsequent research paper published in a peer-reviewed journal, and I declare that I participated in the data generation, analyses and write up of this publication but was not the principal responsible due to the wider scope of the work reported in this publication.

Conference contributions

This paper in Conference Proceedings summarises the research presented in Chapter 2.

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Article in international peer-reviewed journal

This publication includes the data and some analyses presented in Chapter 2.

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Abstract

Lepidoptera is one of the most diverse insect orders found worldwide. African Emperor moths (Saturniidae) are understudied despite their ecological and economic relevance, as the caterpillars of many species are utilized for human consumption. *Gonimbrasia belina* occurs in woodlands of *Colophospermum mopane* (mopane tree) and *Brachystegia* sp. (miombo tree) across southern Africa. Mopane worms, the vernacular name for the edible caterpillars of *G. belina*, is regarded as the most important edible Saturniidae caterpillar in Africa as it provides not only food but also seasonal income for rural communities. As a result, mopane worms are widely harvested in southern Africa due to their high nutrient content and income generating potential. Increased demands for favoured edible Saturniidae species, habitat destruction and unregulated harvesting are placing wild populations in serious danger of decline.

Currently, most research on the genetic diversity of Saturniidae has focused on species farmed in Asia for silk production. African Saturniidae have remained largely unsurveyed, and mitochondrial phylogenies of the family have so far included only *G. belina* and *Gynanisa maja,* two of the most exploited edible caterpillars in southern Africa. This study aimed to bridge this knowledge gap by generating baseline genetic data on seven African Saturniidae species in three tribes: *Heniocha dyops, Gonimbrasia tyrrhea, Bunaea alcinoe, Nudaurelia cytherea* (Bunaeini)*, Epiphora bauhinia* (Attacini)*, Vegetia ducalis* (Micragonini) and *Vegetia grimmia* (Micragonini). For that purpose, I sequenced and described the complete mitogenome of one individual of each species and inferred their phylogenetic relationships with other Saturniidae. The mitochondrial gene content and organisation was conserved across all Saturniidae species in this study. Bayesian Inference and Maximum likelihood phylogenetic reconstructions were performed separately for three datasets: (1) PCG123 - all PCGs and all codon positions, (2) PCG12 - all PCGs with 3rd codon position removed; and (3) PCG123+rRNA – all PCGs and two rRNAs (16s rRNA and 12s rRNA). Previous studies showed similar results, in that none of the phylogenies recovered a monophyletic tribal structure in Saturniini. However, the tribes Attacini, Bunaeini and Micragonini were recovered as monophyletic clades. Additionally, the tribe Micragonini (represented by *Vegetia ducalis* and *Vegetia grimmia*) is here included for the first time in the comparative mitogenomics and mitochondrial phylogeny of the family Saturniidae.

In the context of edible caterpillars of African Saturniidae, special attention was dedicated to *G. belina* because of its large-scale exploitation in southern Africa. I aimed to assess the intra-specific genetic diversity, population structure and phylogeographic structure of the species in South Africa, Namibia and Botswana by analysing two short polymorphic mitochondrial amplicons (Amplicon A; 600 bp; located between ATP6 and COIII, and Amplicon C; 888 bp; located between ND6 and CYTB) in addition to the standard COI barcoding region (700 bp). Overall, *G. belina* had 22 haplotypes and all three counties exhibited low levels of nucleotide diversity and high levels of haplotype diversity, with only one haplotype shared between South Africa and Botswana. Genetic

divergence in *G. belina* corresponds to the broad geographical area of origin of the specimens. A maximum likelihood tree showed that most haplotypes clustered into three groups corresponding to the three countries, evidencing significant phylogeographic structure in *G. belina*. The findings from this study provide valuable information for future studies on the population structure of *G. belina,* and offers baseline data from which biodiversity hotspots may be identified in the future to inform sustainable harvesting and conservations plans in order to preserve this species.

Opsomming

Lepidoptera is een van die mees diverse insekordes wat wêreldwyd voorkom. Ten spyte van hul ekologiese en ekonomiese belang word Afrika-keisermotte (Saturniidae) onderbestudeer. Die ruspes van baie van hierdie spesies word vir menslike behoeftes gebruik. *Gonimbrasia belina* kom voor in *Colophospermum mopane* (mopanieboom) en *Brachystegia* sp. (miombo boom) boslande regoor Suider-Afrika. Mopaniewurms, die volksnaam vir eetbare ruspes van *G. belina*, word beskou as die belangrikste eetbare Saturniidae-ruspes in Afrika aangesien hul nie net kos verskaf nie, maar seisoenale inkomste vir minder bevoorregde gemeenskappe. Dus word mopaniewurms reg oor Suider-Afrika geoes weens hul hoë voedingsinhoud en potensiaal om inkomste te genereer. Verhoogde aanvraag na gewilde eetbare Saturniidae-spesies, habitatvernietiging en ongereguleerde oes plaas wille bevolkings in ernstige gevaar van agteruitgang.

Die meeste navorsing oor die genetiese diversiteit van Saturniidae fokus op spesies waarmee daar in Asië geboer word, vir syproduksie. Daar is ń tekort aan mitochondriale filogenieë van Afrika-Saturniidae, tot dusver het studies slegs twee van die mees gewildste eetbare ruspes in Suider-Afrika, *G. belina* en *Gynanisa maja* ingesluit. Die doel van hierdie studie was om die kennis gaping te oorbrug deur genetiese data oor Saturniidae van Afrika te genereer, deur die filogenetiese posisie van sewe spesies in drie stamme te evalueer: *Heniocha dyops*, *Gonimbrasia tyrrhea*, *Bunaea alcinoe*, *Nudaurelia cytherea* (Bunaeini), *Epiphora bauhinia* (Attacini), *Vegetia ducalis* en *Vegetia grimmia* (Micragonini). Dit het geskied deur, die volledige mitogenoom van ń individu van elke spesie te beskryf en die filogenetiese verwantskappe met ander Saturniidae af te lei. Die mitochondriale geeninhoud en organisasie is dieselfde vir alle Saturniidae spesies in hierdie studie. Bayesiese afleiding en maksimum waarsynlikheid, filogenetiese rekonstruksies word afsonderlik vir drie datastelle uitgevoer: (1) PKG123 - alle PKG's en alle kodonposisies, (2) PKG12 - alle PKG's met 3de kodonposisie verwyder; en (3) PKG123+rRNA – alle PKG's en twee rRNA's (16s rRNA en 12s rRNA). Vorige studies het soortgelyke resultate getoon, deur dat geen filogenieë die monofiletiese stamstruktuur van Saturniini kon ontdek nie. Die stamme Attacini, Bunaeini en Micragonini is egter as monofiletiese klades herwin. Die stam Micragonini (verteenwoordig deur *V. ducalis* en *V. grimmia*) word in hierdie studie vir die eerste keer by die vergelykende mitogenomika en mitochondriale filogenie van Saturniidae ingesluit.

In die konteks van eetbare ruspes van Afrika-Saturniidae, was spesiale aandag aan *G. belina* gegee aangesien hulle die mees misbruikde, eetbare ruspes in Suider-Afrika is. My doel was om die intra-spesifieke genetiese diversiteit, bevolkingstruktuur en filogeografiese struktuur van *G. belina* in Suid-Afrika, Namibië en Botswana te onleetd deur gebruik te maak van twee kort polimorfiese mitochondriale amplikone (Amplikon A; 600 bp; geleë tussen ATP6 en COIII, en Amplikon C; 888 bp; geleë tussen ND6 en CYTB) saam met die standaard COI gebied (700 bp). *Gonimbrasia belina* het al te saam 22 haplotipes bevat en *G. belina* van al drie lande het lae vlakke van nukleotieddiversiteit gehad, hoë vlakke van haplotipe diversiteit. Slegs een haplotipe was

gedeel tussen Suid-Afrika en Botswana. Genetiese differensiasie getoon deur *G. belina* het ooreen gestem met die breër geografiese gebied van waar die monsters vandaan kom. 'n Maksimum waarskynlikheidboom wys dat die meeste haplotipes in drie gegroepeer het wat ooreenstem met die drie lande, en dus aan dui dat *G. belina* filogeografiese struktuur bevat. Resultate van hierdie studie sal waardevolle inligting verskaf vir toekomstige studies oor die bevolkingstruktuur van *G. belina*. Dit bied ń inligtings basis waarvan af gewilde oesstreke in die toekoms geïdentifiseer kan word, en dus sal bydra na die ontwikkling van volhoubare oes- en bewaringsplanne te bewaring van hierdie spesie.

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Abbreviations

%: Percent

°C: Degrees Celsius

3´: Three-prime

5´: Five-prime

A: Adenine

AMOVA: Analysis of molecular variance

ATP6: Adenosine triphosphate synthase subunit 6

ATP8: Adenosine triphosphate synthase subunit 8

BI: Bayesian Inference

BOLD: Barcode of Life Data System

Bots: Botswana

Bp: Base pairs

Bpp: Bayesian posterior probabilities

C: Cytosine

CIPRES: Cyberinfrastructure for Phylogenetic Research COI – Cytochrome oxidase subunit 1

cm: Centimetre

COI: Cytochrome oxidase subunit I

COII: Cytochrome oxidase subunit II

COIII: Cytochrome oxidase subunit III

CYTB: Cytochrome b

d.f.: Degrees of freedom

DNA: Deoxyribonucleic Acid

et al.: And others

F: Forward primer

Fst: Genetic differentiation coefficient

G: Guanine

G: Gram

GHG: Greenhouse gasses

h: Haplotype Diversity

H: Number of haplotypes

HTS: High-throughput sequencing

IGS: Intergenic spaces

K2P: Kimura Two-Parameter model

Kg: Kilogram

Km: Kilometer

M: Meter

MAFFT: Multiple Alignment using Fast Fourier Transform

MEGA-X: Molecular Evolutionary Genetics Analysis

Min: Minutes

ML: Maximum likelihood

mm: Millimetre

mtDNA: Mitochondrial deoxyribonucleic acid

mya: Million years ago

n: Number

Nam: Namibia

ND1: NADH dehydrogenase subunit 1

ND2: NADH dehydrogenase subunit 2

ND3: NADH dehydrogenase subunit 3

ND4: NADH dehydrogenase subunit 4

ND4L: NADH dehydrogenase subunit 4L

ND5: NADH dehydrogenase subunit 5

ND6: NADH dehydrogenase subunit 6

NJ: Neighbour-Joining

NTFP: Non-timber forest product

NUMTs: Nuclear mitochondrial deoxyribonucleic acid

P: P-value

PCG: Protein coding gene

PCG12: All PCGs with 3rd codon position removed

PCG123: All PCGs and all codon positions

PCG123+rRNA: All PCGs and rRNAs (16s rRNA and 12s rRNA)

PCR: Polymerase Chain Reaction

R: Reverse primer

RNA: Ribonucleic acid

rRNA: ribosomal Ribonucleic acid

s: Seconds

SA: South Africa

spp.: Several Species

T: Thymine

Ta: Annealing temperature

tRNA: transfer Ribonucleic acid

UN: United Nations

µL: Microliter

µM: Micromole

π: Nucleotide Diversity

Chapter 1: Literature review

1.1 Introduction

The consumption of insect by humans or entomophagy, has played a valuable role as a source of food for humans through history (Illgner et al., 2016; van Huis, 2013). Emperor moths are of particular importance, with several Asian species (*Antheraea assamensis*, *Antherea pernyi*, *Saturnia japonica*, *Eriogyna pyretorum* and *Samia cynthia ricini*) commercially utilized in silk production (Jiang et al., 2009; Kim et al., 2012). In Africa, Emperor moth larvae are a delicacy and an important source of food as they are high in protein (Hlongwane et al., 2020; Makhado et al., 2014). Additionally, species such as *Epiphora bauhiniae* and *Argema mimosae* have the potential to be the foundation of sericultural practice on the African continent, yet information on African silk-producing species is scarce (Kim et al., 2016; Liu et al., 2020).

Entomophagy is entrenched in human society with insects forming part of an estimated two billon peoples' diets, yet western and westernized societies are reluctant to partake in this practice and have built up an attitudinal barrier against the consumption of insects (DeFoliart, 1999; van Huis, 2013; Anankware et al., 2015). Little do they know that they consume insects daily as a certain number of insects are allowed per 100 grams in processed food products such as chocolate, pasta, wheat flour, peanut butter, spices and frozen vegetables (Yen, 2009a; Anankware et al., 2015). A global food crisis is looming as the human population is project to rise to almost 10 billion by the year 2050 (Jansson & Berggren, 2015). There is no denying that climate change affects the production of food worldwide, in conjunction with urbanization that is claiming an increased amount of arable land needed for food production, at a rapid pace (Yen, 2015). A viable solution to these problems is insect-derived protein. Insects are rich in iron and protein, capable of surviving various ecological conditions, have a high reproductive capacity, short life cycle and favorable feed conversion rates at lower environmental cost compared to conventional livestock (Fanzo, 2013; Van Huis, 2013; Dobermann et al., 2017). Insects therefore have potential to be farmed and treated as "mini livestock" (Elorduy, 2005). Societies worldwide, especially westernized societies, need to make a drastic mind shift to decrease the size of their environmental footprint with regards to food production and acknowledge insects as a food source.

The order Lepidoptera have the largest evolutionary radiation of all herbivorous animals (Nneji et al., 2020; Goldstein, 2017). Additionally, Lepidoptera is the fourth most diverse insects order with more than 180,000 described species worldwide (Huemer et al., 2014; Nneji et al., 2020; Goldstein, 2017). It is estimated that 18% of the 2,000 edible insect species consumed are caterpillars (larvae) of Lepidoptera (Goldstein, 2017). Globally, the family Saturniidae includes an estimated 3,454 species and 180 genera, and in Africa approximately 400 species, 50 genera and six tribes (Saturniini, Bunaeini, Eochronini, Micragonini, Attacini, Urotini) (Hlongwane et al., 2020; Kitching et al., 2018; Huelsenbeck et al., 2013; Sekonya et al., 2020). It has been reported that at

least 50 to 70 caterpillar species of Saturniidae are consumed throughout countries in central, south and western Africa (Hlongwane et al., 2020; Kelemu et al., 2015; Singh et al., 2017). The number of recorded edible caterpillar species increases as new studies emerge. These caterpillars are an inexpensive protein source with high nutritional value, as well as cultural, socio-economic, biological, and ecological value (Makhado et al., 2014; Baiyegunhi et al., 2016). *Gonimbrasia belina,* commonly referred to as mopane worm, is a popular delicacy and is considered one of the most important edible Saturniidae in sub-Saharan Africa (Ghazoul, 2006; Hope et al., 2009). Taxonomic inconsistencies and instability in the Saturniidae family have resulted in difficulty determining the exact number of species (Kitching et al., 2018; Hurst et al., 2020). Allocating the wrong species name to specimens results in the number of Saturniidae species occurring and consumed in Africa being overstated or understated, which in turn impacts the implementation of conservation strategies (Hurst et al., 2020).

A sharp decline has been observed globally in saturnid populations that are of importance to humans (Chen et al., 2020; Ghazoul, 2006). The concern is that the biodiversity of this family will disappear before it is fully documented, due to slow taxonomic progression (Yen, 2015). Various studies have reported a serious decrease of wild *G. belina* populations, raising doubt on the sustainability of this seasonal natural resource in southern Africa (Illgner et al., 2016; McGeoch, 2002; Roberts, 1998). Anthropogenic factors such as overharvesting, habitat destruction, pollution, and fires, together with natural pressures such as predation and drought are likely at the root of this decline (Yen, 2015). In some areas, larval production has been severely compromised, and the chances of the population recovering naturally is very slim (Illgner et al., 2016; Roberts, 1998). However, it is evident that the mopane worm trade not only alleviates nutritional deficiencies but is a source of economic empowerment in rural communities on the African continent (Ramos, 2005; Hurst et al., 2020).

Despite the important cultural, economic and ecological role played by edible caterpillars across the African continent, there are limited studies on evolution of, genetic diversity, phylogeography and population structure of these Saturniidae. Additionally, Saturniidae mitochondrial phylogenies have predominantly been limited to Asian species used in sericulture (Chen et al., 2020; Jiang et al., 2009; Kim et al., 2018; Kim et al., 2009). A recent study by Langley et al. (2020) added the first African species (*G. belina* and *Gy. maja,* both in the tribe Bunaeini) to the mitochondrial phylogeny of saturnids (Langley et al., 2020). However, *G. belina* and *Gy. maja* mitogenomes only represent approximately 1% of the total Saturniidae species recorded in Africa and 4% of all saturnid mitogenomes publicly available (Oberprieler, 1977). The only other study done on African Saturniidae at the molecular level was performed in 2001, on allozyme variations in two *G. belina* populations to determine if limited flight ability affects gene flow and if gene expression differs in different stages of their life cycle (larvae, pupae, and moths) (Greyling et al., 2001). The limited availability of baseline genetic information on African Saturniidae is an obstacle that hinders the evaluation of phylogeographic structure, phylogenetic relationships and genetic diversity of species

in this family. Thus, genetic data is required to fill this knowledge gap and asses the current diversity of Southern African edible caterpillar species and populations.

This study assessed the phylogenetic position of seven species in three tribes: *Gonimbrasia tyrrhea, Heniocha dyops, Nudaurelia cytherea, Bunaea alcinoe* (Bunaeini)*, Epiphora bauhinia* (Attacini)*, Vegetia ducalis* (Micragonini) and *Vegetia grimmia* (Micragonini) therefore generating baseline genetic data on African Saturniidae. For that purpose, we sequenced and described the complete mitogenome of one individual of each species and inferred their phylogenetic relationships with other Saturniidae.

Additionally, polyphagous species like the widely distributed *G. belina*, feed on various hosts, therefore, have a high potential to be farmed, thus protecting wild populations. *Gonimbrasia belina* caterpillars are extensively harvested, traded as well as consumed by both rural and urban populations in South Africa, Botswana and Namibia. Yet limited genetic data was available on the intraspecific genetic diversity, phylogeography, population structure and gene flow of this species. Special attention was dedicated to *Gonimbrasia belina* in this study, because of its large-scale exploitation in southern Africa

1.2 Biology and ecology

Emperor moths belong to the family Saturniidae and are named after the planet Saturn, as coloured rings on their wings form eyespots that resemble the rings around Saturn (Pinhey, 1972). These large moths have wingspans of 120 mm to 150 mm and act as health indicators for specific habitats (Ditlhogo, 1996; Oberprieler, 1997). In general, Emperor moths are nocturnal and during the day they rest among foliage or on tree trunks, where they are well camouflaged (Ditlhogo, 1996). Emperor moths camouflage themselves by pulling their forewings over the eyespots on their hindwings (Oberprieler, 1997). To deter predators, moths jerk up their forewings exposing the large eyespots, when threatened. Some species, such as *Gy. maja*, enhance that effect by flapping their wings periodically to mimic the way owls open and close their eyes (Oberprieler, 1997). Emperor moths do not fly throughout the night but instead each sex has definite flight periods, which is species specific (Veldtman et al., 2007). Flight serves a different purpose for male and female moths. Males fly before mating to search for a female to mate with, while females only take flight to lay their eggs, thus flying after mating has occurred (Veldtman et al., 2007). Male moths can fly further than their female counterparts therefore are able to mate with females from another sub-population which influences gene flow between populations (Veldtman et al., 2007). The feathery antennae of male moths aid male Emperor moths in their search for a mate, by detecting sex pheromones excreted by female Emperor moths (Klok & Chown, 1999).

1.3 Life cycle of Emperor moths (*G. belina*)

The most utilized African Emperor moth is *G. belina* caterpillar commonly known as the mopane worm. The Emperor moth's life cycle from egg to adult is about three months, with the average lifespan of an adult only lasting three to four days (Klok & Chown, 1999). Emperor moths have non-functioning mouth parts (atrophied) thus they are unable to feed in the adult stage (Oberprieler, 1997). Female Emperor moths only mate once in their short lifespan, whereas males mate up to five times (Oberprieler, 1997). At the start of the breeding season, female moths oviposit single egg clusters of 30 to 300 on twigs and leaves of mopane trees (*Colophosperumum mopane*) (Ditlhogo, 1996). Larvae hatch approximately seven to 15 days after ovi have been deposited (Ditlhogo, 1996). The larvae take six weeks to pass through five instar stages and is followed by pupation, which concludes the breeding season (Hope et al., 2009). Larvae in instars one to three forage together in groups of 20 to 200 individuals to help regulate their body temperatures, decrease water loss and for protection against predators (Klok & Chown ,1999). Larvae do not bore into mopane trees but instead feed exclusively on the trees leaves (Illgner et al., 2016). Fifth instar larvae are more solitary as they prepare to change into an immobile pupa (Hope et al., 2009). Most African Emperor moths, except *E. bauhinia* have lost the ability to spin silk cocoons but rather pupate in a subterranean cell, at this stage the complete transformation to adult moth occurs (Ghazoul, 2006). Pupation typically occurs for six to seven months in the winter, whereafter the adult moth emerges at the beginning of summer (Ghazoul, 2006; Hope et al., 2009). A combination of various factors including rainfall, temperature changes, humidity and daylength are suspected to trigger eclosion (Togarepi et al., 2020). Rainfall is an important factor for species who pupate underground as without rain the soil will be too hard for the moth to emerge. In more tropical environments, mopane worms produce two generations per rainy season (are bivoltine), the first emergence in December and January, while the second occurs between April and May if enough rainfall occurs (Ditlhogo, 1996; Kopij, 2014). In more arid and semi-arid regions like Namibia they are univoltine (produce a single generation per rainy season) only emerging once annual rainfall occurs (Kopij, 2014).

1.4 Distribution and habitat

Contrary to popular beliefs, the mopane worm (*G. belina*) distribution is not confined to the distribution of *C. mopane* (mopane trees), but it does closely follow their distribution throughout Southern African countries like Malawi, Namibia, Botswana, Zimbabwe, Mozambique and South Africa (Baiyegunhi et al., 2016). *Gonimbrasia belina* is a polyphagous species feeding on a variety of hosts like *C. mopane*, *Carissa* spp., *Diospyros* spp., *Ficus spp*., *Searsia* ssp., *Sclerocarya caffra*, *Terminalia* spp and *Trema* spp (Thomas, 2013). Mopane worms and their various host trees share a co-evolutionary relationship, where the host trees provide food for the mopane worms and the worms feces provides nutrients for the trees. (Ditlhogo, 1996). Mopane worm caterpillars can be

identified by their red, white, yellow, and black colour with distinct short black spikes. Caterpillars feed day and night storing energy needed for the next larval stage. Caterpillars will only leave the tree on which their eggs were laid if that tree is so overpopulated that it is entirely defoliated before the caterpillars complete their larval stage (Pillay, 2015).

Gynanisa maja, the speckled Emperor moth or more commonly known as the green mopane worm, is a polyphagous species found on an assortment of host plants from the genera *Acacia* and *Querus* to mention a few (Togarepi et al., 2020). Like *G. belina,* they are widespread and distribution ranges from as far north in Africa as Chad to as far south in Africa as Namibia and South Africa (Kopij, 2014).

Another relative of *G. belina* and *Gy. maja* (Bunaeini) is *Gonimbrasia tyrrhea,* commonly known as the Zigzag Emperor moth (Pinhey, 1972; Pinhey, 1956). Zigzag Emperor moths are identified by a black zigzag pattern on their forewings (Pinhey, 1956). They are yet another polyphagous Saturniidae species with this species being found on a multitude of host plant from the genera *Eucalyptus* and *Acacia* spreading from south to east Africa (Pinhey, 1956).

Nudaurelia cytherea, the Pine Tree Emperor moth is another member of the Bunaeini tribe. Pine Tree Emperor caterpillars are known as agricultural pests as they defoliate pine trees throughout their distribution range (Geertsema ,1971). The adult moths are yellow with eyespots on their forewings and are found on a multitude of indigenous host plants in south and eastern Africa (Pinhey, 1972).

The Cabbage Tree Emperor moth, *Bunaea alcinoe* also belongs to the Bunaeini tribe which occupies a diverse biome range throughout the African continent (Pinhey, 1972). Their nutritious caterpillars are easily identifiable by their black bodies, and ivory and orange spines (Pinhey, 1956). The colours of adult moths vary according to the environment where they are found and range from purple-brown to orange-brown (Cooper, 2002).

Heniocha dyops, the Western Marbled Emperor moth (Bunaeini) has a wide distribution range in southern Africa where they can predominately be found on host plants from the genera *Acacia* in Angola, Zambia, South Africa, Namibia, Botswana and Democratic Republic of Congo, to mention a few (Pinhey, 1972). The adult moths can be identified by their white colour and forewings that have a brown eyespot. Caterpillars are green, with short ivory and violet spines and a yellow line running laterally on either side of their bodies.

Epiphora bauhinia, the Southern Atlas Emperor moth (Attacini) is mainly distributed throughout south and eastern Africa but are also known to occur as far west as Gambia, Senegal, Guinea, and Sierra Leone (Pinhey, 1972). Their larvae generally prefer to feed on host plants from the genus *Ziziphus* (Cooper, 2002).

Vegetia ducalis (Ducal Princeling) and *V. grimmia* (Grim Priceling) are both in the tribe Micragonini. African Saturniidae have been severely understudied, therefore there is limited information on the

distribution and preferred host plant for both these species (Pinhey, 1972). However, specimens for this study were collected in the Western Cape province of South Africa from the host plant *Eriocephalus africanus*.

1.4.1 Factors influencing availability and distribution of African Saturniidae

1.4.1.1 Climatic influences

The amount of Emperor moths that survive their complete life cycle (from egg to adult) is influenced by a combination of climatic and non-climatic factors. Climatic factors including rainfall, favorable temperatures and vegetation availability are important environmental factors which play a role in facilitating egg laying and significantly impact the distribution and availability of Saturniidae (Baiyegunhi et al., 2016; Kopij, 2014; Sekonya et al., 2020; Togarepi et al., 2020). Although Saturniidae can survive both high and low temperatures, extreme heat can cause eggs to burst (Braide et al., 2011). Low and erratic rainfall not only affects the breeding of Emperor moths, but also the availability of host plants (Braide et al., 2011; Kopij, 2014; Sekonya et al., 2020). Additionally, high rainfall and flooding negatively impact Saturniidae populations. Heavy rainfall cause caterpillars to fall off trees and drown in the water, if they cannot burrow into the ground to pupate (Thomas, 2013).

1.4.1.2 Non- climatic influences

Anthropogenic factors have the greatest influence on the decline of African Saturniidae, especially edible species (Dube et al., 2013). One of the major anthropogenic factors which impact African Saturniidae populations is deforestation through collection of firewood, materials for construction and fencing, as well as clearing natural vegetation for crop production (Pillay, 2015). Consequently, when larval food plants are destroyed, the breeding of the saturnids will be affected. Additional factors such as urbanization further contribute to the decline of African Saturniidae (Dube et al., 2013). Mining activity causes dust to accumulate on the leaves of host plants making them toxic, which in turn affects the egg laying of Emperor moths (Pillay, 2015). Over-harvesting of edible African Saturniidae such as mopane worms has also contributed to the significant decline of these populations (Sekonya et al., 2020). Mopane worms have traditionally been harvested sustainably but the increase of their economic value has led to a drastic increase in harvesting. Desperate harvesters follow destructive harvesting practice such as cutting of branches and even whole trees to access caterpillars (Lautenschläger et al., 2017). Additionally, populations are negatively impacted by greedy harvesters which harvest so many caterpillars in a particular area that it affects the next generation by decreasing the population numbers to such an extent that harvesting in the same region is no longer feasible (Togarepi et al., 2020). The lack of harvesting governance and the failure to implement harvesting guidelines contributes to the decline seen in edible African saturnids (Sekonya et al. 2020).

In addition to anthropogenic factors, copious natural predators such as parasites, birds, insects, monkeys, reptiles, bats, jackals and warthogs prey on different stages of the mopane worms life cycle (Thomas, 2013). Soil type also influences how many larvae pupate successfully; thus, representing a determining factor of mopane worms' pupal distribution (Ghazoul, 2006). Larvae that pupate in sandy soil have higher survival rates, as this type of soil has low water retention capacity therefore decreasing the risk of pupa drowning (Ghazoul, 2006). Sandy soil is softer and therefore more suitable for burrowing of fifth instars and emergence once the pupation stage has been completed (Ghazoul, 2006). Sandy soils microclimate does not promote the growth of harmful bacteria and fungi which decrease the survival rate of pupa (Ghazoul, 2006). Additionally, host characteristics and habitat structure are also important factors in the life cycle of various saturnids (Veldtman et al., 2007). Key characteristics of host trees include canopy size, tree connectivity, tree size and tree density (Khaliq et al., 2015; Veldtman et al., 2007). High larval population densities are observed and connected to tall trees with large canopy sizes as more food is available and the risk of attack from predators is decreased (Khaliq et al., 2015).

1.5 Entomophagy

1.5.1 Benefits of entomophagy

Malnutrition is the cause of half the deaths of children under five worldwide. Many developing countries rely on insects as a source of protein, fiber, healthy fat, vitamins and minerals (van Huis, 2016). The most consumed insect species include locusts, beetles, bees, ants, crickets and caterpillars of Lepidoptera (Chakravorty, 2014). Food production is one of the major contributors to climate change, responsible for approximately 30% of global greenhouse gasses (GHG) emissions (Sasson, 2012; Rettore et al., 2016). Insect farms have a much smaller environmental footprint as they emit less GHG and require much less land and water than cattle farms (van Huis, 2016).

There are currently seven billion people on planet Earth and even though we produce more than enough food to feed everyone, one in seven people goes hungry (Sasson, 2012). Meaning that one billion people worldwide do not have access to proper nutrition to live a healthy life (Sasson, 2012). This is important as the United Nations (UN) has predicted that by the year 2050 the world population will reach 9.7 billion people (Huelsenbeck et al., 2013). This means that food production needs to double to feed all people. There is simply not enough agricultural land to produce these astronomical volumes of food and feed (Kelemu et al., 2015).

Insects are rich in macronutrients such as fat, protein and fibre. The nutritional value of insects depends on the harvested insects' metamorphic state, species, diet and preparation (dried, blanched or fried) (Kauppi et al., 2019). Insects farmed for human or animal consumption can therefore be fed a modified diet that results in optimal nutritional value (Glover & Sexton, 2015). The building blocks of protein are amino acids, animals cannot synthesize all the essential amino acids (Diehl et al., 2014). Several essential ammino acids are therefore required in animal feed for

optimal growth (Diehl et al., 2014). Amino acid requirements differ among different livestock animals, therefore several insect species could be mixed to produce the most nutritious feed for specific livestock animals (Diehl et al., 2014; Khan, 2018). Insect meal contains amino acids which promotes the production of milk, foetal growth and strong immune systems in mammals (Madibela et al., 2009). Additionally, insect-based feed can be used together with traditional animal feed (Khan, 2018).

Protein derived from insects is regarded as the most sustainable animal protein source for both human and animal consumption (Kauppi et al., 2019). Humans, especially in western and westernized societies are currently not ready to make insects a part of their daily diets (Rettore et al., 2016). The increased demand for protein from an increasing global population has led to an increased demand for animal feed. The food supply chain is therefore under severe pressure with the increased demand for animal feed and limited availability of fishmeal as result of pollution and over exploitation of marine resources. Furthermore, the lack of available land has limited the availability of soy beans and maize, and has further strained the already severely pressured food supply chain (Rettore et al., 2016). Insect as animal feed is a more sustainable and economical feed source than conventional feed sources such as fishmeal, soybeans, maize and other grains, especially because the natural diets of poultry and fish are comprised of insects (Diehl et al., 2014; Khan, 2018; Glover & Sexton, 2015). Additionally, dried insects have a crude protein content between 30% and 70%, which is the same or higher than the crude protein content derived from soybean and fishmeal (Diehl et al., 2014).

Insects gain body weight quickly, are small in size and grow optimally regardless of density as long as sufficient food is available, therefore insect rearing requires less space (Müller et al., 2016; Kauppi et al., 2019; Rettore et al., 2016). Insects have a favourable feed to meat conversion rate when compared to that of conventional protein sources. To produce 1 kg of meat crickets require 1.7 kgs of feed compared to beef which requires 10 kgs of feed, pork 5 kgs and chicken 2.5 kgs of feed (Kauppi et al., 2019). The agricultural industry uses 70% of the global fresh water supply (Kauppi et al., 2019). To produce 1 kg of beef approximately 15,000 litres of water is used (Müller et al., 2016). It is clear that we need to make considerable changes to our global food supply chain in order to make it more sustainable (Rettore et al., 2016).

Organic waste from restaurants or kitchen leftovers can be recycled and used as feed for insects thus making insect feed a more cost efficient and environmentally friendly protein sources (Kauppi et al., 2019). A shift from traditional protein sources as food or feed could result in natural resource being used more efficiently and lower emission of GHG (Khan, 2018). Entomophagy could therefore contribute a sustainable solution to the eminent food shortage that is looming (Yen, 2015).

1.5.2 Problems associated with entomophagy

Insects have an exoskeleton and segmented body (like crustaceans) and are classed as arthropods (Hebert et al., 2003). A concern when consuming insects is that it may trigger an allergic reaction like that of shellfish (Dobermann et al., 2017). Even though insects have been consumed for centuries by various cultures worldwide, a lot of information on how consuming insects may affect our bodies is still unknown (Egan, 2013).

The exposure to harmful bacteria is a concern when consuming insects especially those caught in the wild. Various Insects species feed on decaying matter like animal corpses, rotten food and human waste, the perfect environment for bacteria (Dobermann et al., 2017). To offset this problem insect farms, need to abide by high hygienic standards and provide a clean environment to rear insects suitable for human consumption (Dobermann et al., 2017). More research needs to be done before insects can be safely introduced as a dietary staple.

Anti-nutrients are substances that inhibit the body's ability to absorb and utilize protein (Fanzo, 2013). They are commonly found in foods made from plants like rice and flour (Fanzo, 2013). The most common anti-nutrients are phytic acid, lectins and tannins (Madibela et al., 2009). Insect exoskeletons are made of chitin which contains small amounts of anti-nutrients (Egan et al., 2014). Chitin may inhibit the body's ability to absorb protein and high concentration may induce asthma attacks (Dobermann et al., 2017). Conversely, the health benefits of chitin consumption include increasing the body's immune defense, decreasing inflammation and combating cancer (Dobermann et al., 2017). Only about 2% of insects dry body mass is comprised of chitin (Diehl et al. 2014). Many studies have shown that anti-nutrients in edible insects are low when compared to plant-based food, yet more research needs to be done to determine their significance.

Energy usage of mass-reared insects are similar or even less efficient when compared to that of traditional protein sources such as pork and chicken. Mass rearing insects requires rooms heated to temperatures which encourage optimal growth and weight gain (Diehl et al., 2014). In addition freezing, oven drying and grinding of insects to produce insect feed consumes a sizeable amount of energy which has numerous impacts on the environment (Diehl et al., 2014).

There is not much research on pesticides used when rearing insects. However, one study did find low levels of harmful chemicals, no greater than that found in plant or animal-based foods (Shadung, 2012). It cannot be ignored that pesticides used in edible insect production is unregulated, therefore pesticide exposure is a risk associated with entomophagy (Dobermann et al., 2017). The risk of pesticide contamination is greater for insects harvested from wild populations, than those farmed responsibly.

Certain insects carry toxins to protect themselves from predators, making them toxic if consumed (Malaisse & Latham, 2014). Certain toxic and edible insects are morphologically similar, one can

make you very ill while the other is a nutritious food source (Yen, 2015). People need to keep this in mind and not eat just any insect (Yen, 2009b).

The main takeaways regarding the safety of insect consumption as food or feed are sourcing good insects, being cautious if your allergic to shellfish, do not just eat any insect it may be toxic and ensuring that insect feed is administered in pellet form and not powdered form (to prevent the inhalation of chitin) (Diehl et al., 2014; Dobermann et al., 2017).

1.5.3 Entomophagy in Africa

1.5.3.1 African Saturniidae as a food source

The African continent has the highest malnutrition and lowest daily protein intake per capita (8 g) compared to developed regions such as North America, which has the highest daily protein intake per capita (71.2 g) (Sasson, 2012). Low protein intake in Africa is linked to low income in rural areas, which limits the access to conventional animal protein sources (Siulapwa et al., 2012). Thus, the importance of alternative protein sources for both humans and animals must be highlighted. Insects are highly nutritious and have a high feed conversion rate with relatively low environmental cost (Obopile & Seeletso, 2013). Insect therefore can provide affordable essential nutrients to people who do not have access to or cannot afford conventional protein sources such as beef, poultry, pork and fish (Sasson, 2012). Entomophagy is a common practice in developing countries as edible insects are affordable nutritional supplement, particularly in times of crisis (Ebenebe et al., 2017). In sub-Saharan African countries such as South Africa, Malawi, Botswana, Namibia, Zimbabwe and Zambia, the larval stages of at least 50 African Saturniidae species are consumed (Pinhey, 1972; Cooper, 2002).

Knowledge is the foundation of entomophagy as edible insects are selected by different traditional societies based on taste, traditional beliefs, region and seasonal availability (Ebenebe et al., 2017). Thus, certain species are viewed as edible by one group of indigenous people and not by another. The most significant caterpillar species in southern Africa in terms of trade, volume and consumption is the caterpillar of *Gonimbrasia belina* (mopane worm) (Lautenschläger et al., 2017). It provides important seasonal income from December to January and March to April for many rural communities (Ebenebe et al., 2017). Insects' nutritional value varies with each stage of metamorphosis, additionally different processing and preparation methods also affect the nutritional value (Kelemu et al., 2015). Dried mopane worms have approximately three times the amount of protein per 100 g (56.8 g) than beef (20 g) (Mutungi et al., 2019). Additionally, they have a high energy content providing 444 calories of energy per 100 g. In comparison, beef only provides 250 calories per 100 g, half the amount provided by dried mopane worms (Mutungi et al., 2019). Dried mopane worms can be stored for months, making it a valuable food source during the dry season when food is scarce. However, when stored or processed incorrectly dried mopane worms provide an environment that encourages growth of pathogenic micro-organisms such as

fungi (Mutungi et al., 2019). Subsequently, micro-organisms such as *Aspergillus flavus* and *A. parasiticus* can spoil the dried mopane worms and expose people to toxins such as aflatoxins (Braide et al., 2011). Aflatoxin toxicity causes nauseas, vomiting, abdominal pain, in severe cases cause convulsions and acute liver damage (Braide et al., 2011). Adequate storage and processing of mopane worms is essential. It is therefore recommended to store mopane worms in a fridge or freezer and if cold storage is not available, drying in the sun or boiling in a vinegar solution before storing is recommended to prevent microbiological contamination (Hanboonsong & Durst, 2014). In addition to normal hygienic precaution used when preparing food, it is recommended to degut and then boil or fry mopane worms to reduce microbiological contamination and make them safe for human consumption (Hanboonsong & Durst, 2014).

1.6 Harvesting and trading of mopane worms

Mopane worms are a significant and valuable non-timber forest product (NTFP) for rural communities established close to mopane woodlands (Ghazoul, 2006). Mopane worms are traditionally harvested as an important seasonal food resource (Ghazoul, 2006). In southern Zimbabwe, approximately 79% of households barter or sell harvested mopane worms to meet their needs (Gondo et al. 2010). Hence, mopane worms are a popular delicacy in rural households in Southern African countries such as Zimbabwe, Botswana, South Africa, Namibia and Zambia (Illgner et al., 2016). These caterpillars are sold to generate income therefore are economically important. This has resulted in intense international trade of dried mopane worms between African countries to meet the high demand for this resource consumed by humans and used as animal feed (Makhado et al., 2014). It is estimated 9.5 billion mopane worms are harvested per annum in sub-Saharan Africa (Halloran et al., 2014). Approximately 16,000 tons of dried mopane worms are exported to Zambia and Zimbabwe from Botswana and South Africa (Ghazoul, 2006; Hope et al., 2009; Thomas, 2013). The cross-border trade between these African countries was valued at between R422 million and R638 million per annum (Makhado et al., 2014). In 2013, a 50 kg maize meal sack of mopane worms could be purchased in Namibia for about R716.00 (Thomas, 2013). Makhando et al. (2014) reported that a 80 kg maize meal sack of mopane worms would have cost between R752.26 and R1,000.98 in 2005. At the Thohoyandou markets in the Limpopo province of South Africa, a 80 kg mopane worm filled maize meal sack can be purchased for R1,200.00 - R1,500.00 (Makhado et al., 2014). Thohoyandou market vendors reported annual turnovers of up to R20,000.00 from mopane worm sales (Makhado et al., 2014).

Consequently, the high demand for mopane worms, has resulted in a shift from traditional harvesting to commercially-driven harvesting. Traditionally, harvesters were people living near or within outbreak areas (Roberts, 1998). Nowadays harvesters travel up to 250 km or more to outbreak areas (Togarepi et al., 2020). At the time that emergence occurs, hundreds of harvesters hand-pick mopane worms off the trees. Overexploitation of this valuable resource is a major concern and a notable decline in mopane worms' availability has been observed over last 15 years

(Roberts, 1998). This decline is intensified by competition between harvesters. Mopane worms are traditionally harvested during the fifth instar stage of their life cycle, as the caterpillar are fully grown and have minimum or no plant material in their gut making the degutting process effortless (Madibela et al., 2009). A result of inexperienced harvesters and increased competition between harvesters, it has become a more common practice for harvesters to target caterpillars in the third or fourth instars (van Huis, 2013).

1.7 Lepidoptera farming

Insect farming requires insect to be bred in enclosure in order to increase productivity (van Huis, 2016). Farmed edible insects' carbon footprint is considerably lower compared to that of livestock and other protein sources (Sasson, 2012). Additionally, insect farms need less water, have low start-up costs, and need less space for breeding as the life cycle of insects is considerable shorter than that of conventional livestock (Ebenebe et al., 2017). Insects are small and their size allows for both home and industrial rearing therefore providing opportunity for household income and innovative business strategies (Kauppie et al., 2019).

The first lepidopteran species to be farmed was the mulberry silkworm (*Bombyx mori*) (Jiang et al., 2009; Singh et al., 2017). The 5,000-year-old Chinese silk industry has a dual purpose as the by-products of this industry (silkworm pupae) are sold as food (Nneji et al., 2020; Yen, 2015). *Bombyx mori* pupae contain 61% protein, 8% fat and 389 calories per 100 g dried mass (Kauppi et al., 2019). The silk industry has therefore led to well-established lepidopteran farms in Asia. Only a few Lepidoptera species (silk producers) are fully domesticated, although many more edible lepidopteran species have the potential to be farmed (Nneji et al., 2020). A lack of lepidopteran farms, especially in Africa is linked to the absence of: identification methods, basic ecological and biological information, and knowledge regarding the extent to which humans consume them (Malaisse & Latham, 2014).

The mopane worm (*G. belina*) is a vital source of protein and food source in Africa (Siulapwa et al., 2012). A 100 g of dried mopane worms contain 50 to 60 g of protein, providing 76% of an adult's daily protein requirements and more than a 100% of daily vitamins and minerals requirements (Siulapwa et al., 2012). Mopane worms are currently harvested from the wild. Some harvesters have started to implement semi-domestications techniques as *G. belina* emergence seems to depend on multiple climatic and biotic factors, thus making them very unpredictable (Togarepi et al., 2020).

Local harvesters have started semi-domesticating all life stages of *G. belina* from eggs to adults in an attempt to conserve wild populations and possibly take the first step in domesticating mopane worms (Hope et al., 2009; Riggi et al., 2016). Semi-domesticating also makes the irregular and unpredictable nature of mopane emergance more predictable and regular (Hope et al., 2009). Methods involve egg collection and storage or protection by placing a chiffon sleeve over mopane

or host tree branches until eggs are hatched (Hope et al., 2009). Once hatched larvae are protected from natural predators and parasites by placing a shade cloth over host plant on which the larvae feed, as well as installing bird deterrents (Hope et al., 2009). Host plants in the area are monitored so caterpillars feeding on a overpopulated host plant are moved to a less populated host plant, as this helps to decrease competition for food and prevents host plants from being defoliated (Hope et al., 2009). The majority of fifth instar caterpillars are harvested and processed for consumption and 10% of larvae are left to complete their life cycle to keep population numbers stable (Hope et al., 2009). Caterpillars ready to pupate are transferred to pupation pits, and prior to the moths pupating, pupae are transferred to carboard boxes (Hope et al., 2009). Once the moths emerge from their pupae, they are released under a protective shade cloth at the base of host plants, this allows male and female moths to mate and the whole life cycle to repeat (Riggi et al., 2016).

1.8 Factors impacting the implementations of conservation strategies for African Saturniidae

In countries where wild edible insects are a staple food source, nature conservation is extremely important. Especially when edible insects are not only a staple food sources for the local communities but also have medicinal and spiritual uses (Yen, 2009a; Yen, 2015). Unfortunately, most conservation strategies fail to integrate cultural and biological conservation (Halloran et al., 2015). In southern Africa, a sharp decline in wild *G. belina* populations has been observed over the last decade (Thomas, 2013). The high demands for this delicacy has resulted in overharvesting to supply a complex network of commercial markets involving national and international trading of mopane worms (Lautenschläger et al., 2017; Makhado et al., 2014).

The identification, location of emergence and collection techniques of edible insects are passed down from generation to generation (Ebenebe et al., 2017; van Huis, 2016). Traditional knowledge is thus vital to the sustainable utilization of edible African Saturniidae species, especially as adequate scientific knowledge is lacking for these saturnids (Kelemu et al., 2015). It is therefore essential that traditional knowledge is recorded and used to help up lift the community. Recording ethnic or traditional knowledge is a complex process as ethnic names for edible Saturniidae may differ from one community to another (Bomolo et al., 2017). One community might consider a certain saturnid species as edible while another considers that same saturnid species inedible. Genetic data is thus essential in providing taxonomic clarity and correct species identification of edible species from traditional knowledge (Hebert et al., 2003; Silva-Brandão et al., 2009; Oberprieler, 1977). The correct identification of edible saturnids is essential information which is needed to develop regional sustainable management plans for these species (Bomolo et al., 2017; Sarre & Georges, 2009). Additionally, genetic studies on edible Saturniidae species will provide crucial information on population dynamics from which more sustainable harvesting methods can

be developed. Genetic studies will assist in identifying genetic variation in population from different region which therefore could provide information from which harvest site locations could be determined (Sarre & Georges, 2009).

Food safety concerns of edible insects overshadow the importance of nature conservation, food security, potential economic development and traditional food culture (Halloran et al., 2015). The absence of inclusive legislations that governs the use, trade and production of insects as a source of food and animal feed has delayed the development of the edible insect sectors (Halloran et al., 2015). There are hardly any examples of national regulation that govern entomophagy despite it being a common practice in several regions worldwide (Halloran et al., 2015). Measures such as government legislation and permits need to be implemented to control harvesting of *G. belina* in Africa (Sekonya et al., 2020). Permits will aid in the regulation and monitoring of the number of harvesters, size and number of caterpillars allowed to be harvested, and number of days harvesting is allowed (Jones, 1999). Permits will regulate which outbreak areas can be harvested, therefore allowing outbreak areas to be harvested on a rotational basis, and consequently allowing the recovery of populations in over harvested areas (Sekonya et al., 2020). Habitat loss is one of the greatest threats to biodiversity, therefore protecting *G. belina* host plants in outbreak areas is crucial (Halloran et al., 2015; Hanboonsong & Durst, 2014). In Africa, permit governance is weak especially in regions where lands are managed by the local community and property boundaries lack physical makers such as fences (Sekonya et al., 2020). When developing future legislations, it is imperative to consider the multi-dimensional nature of entomophagy (Halloran et al., 2015), especially because entomophagy could be a vital pathway to ensuring food and feed security (Diehl et al., 2014; Glover & Sexton, 2015; Kauppi et al., 2019; Rettore et al., 2016).

Semi-domestication refers to the manipulation of natural environments to protect local populations. Semi-domestication involves habitat manipulation, creation of a habitat and translocation of individuals to a more favorable breeding site (Hope et al., 2009; Riggi et al., 2016). Methods includes translocation of eggs and caterpillars to host plant closer to harvesters' homes, protection of eggs by mesh bags, planting and conserving host plants near villages (Hope et al., 2009). Planting host plants not only provides a breeding habitat but the potential of multiple other agroforestry opportunities (Vantomme et al., 2004). In Tanzania researchers saw a link between profitable butterfly farms and effective forest conservation (Temitope et al., 2014). Semi-domestication has proven successful in conserving *G. belina* populations numbers in various Southern African villages which have implemented it (Hope et al., 2009). Since small-scale farming uplifts the community and large-scale farming conditions increase the risk of viral and bacterial infection, it is therefore more useful to have several small farms than one large area or farm, therefore possibly providing a whole village or community with an extra or alternative source of income and resulting in the economic security for rural communities (Ghazoul, 2006; Hope *et al.,* 2009).

1.9 Classification

1.9.1 Morphological identification

Taxonomists specialize in classify organisms by naming and describing species by means of morphological, biochemical and behavioral observations (Silva-Brandão et al., 2009). Specimens received by taxonomist are roughly grouped into species, thereafter the hard work begins. Taxonomist then need to corroborate their identification by reading identification guides, some of which were written 200 years ago, and borrowing named museum specimens for comparison (Oberprieler, 1977). Traditional taxonomic identification is a time-consuming process and additionally there is a major shortage in trained taxonomist worldwide. It is believed that there are more unknown species than known species on earth, suggesting that millions of species are yet to be described and there are far too few taxonomists to do it (Silva-Brandão et al., 2009).

Insects are an important ancient taxonomic group and are distributed worldwide (Packer et al., 2009). Insects are very diverse with various insects being considered as pest, vectors that carry disease, parasites, pollinators and bio-indicators. Insects are therefore an essential part of the food chain. Species identification solely based on the morphological characteristics of insects is difficult (Packer et al., 2009). Since insects of the same species may be morphological different due to sexual dimorphism or different developmental stages of individuals. There is a lack of user-friendly identifications guides for non-taxonomist (Packer et al., 2009).

1.9.2 Molecular identification

The insect mitochondrial genome, like most animals, is a double stranded molecule, which ranges in size from 15,000 to 19,000 bp (Boore, 1999). It is comprised of 37 genes of which 13 are protein coding, 22 transfer RNAs (tRNA) and two ribosomal RNAs (12s and 16s) (Bao et al., 2019). Additionally, it also contains a non-coding AT rich region, where the initiation of transcription and replication is located (Bao et al., 2019). The mitochondrial gene arrangement is highly conserved in animal phyla (Kim et al., 2012). Even in insects, which have highly variable mitochondrial gene arrangements, these generally only affect transfer RNA (tRNA) genes and rarely protein coding (PCG) and ribosomal RNA (rRNA) genes (Giegé et al., 2015).

The mitochondrion plays a crucial role in ageing, respiration, genetic illness and self-destruction, often being referred to the cell's powerhouse (Ross, 2014). Mitochondrial DNA (mtDNA) is the genetic material in mitochondria and produce enzymes for protein synthesis and oxidative phosphorylation (Boore, 1999). Currently, the mitochondrial genome is viewed as the most important genomic resource in systematic entomology (Ross, 2014). The mitochondrial genome is useful in phylogenetic reconstructions as it has a conserved simple structure, with strict maternal transmission and a high mutation rate (five to 10 times that of nuclear DNA) due to a limited repair system (Wolff et al., 2012). Mitochondrial DNAs' unique properties allow for development of

universal PCR primers across a wide range of species (Lemire, 2005). Additionally, high numbers of mitochondria in most cells allow for easy recovery of DNA from samples with degraded DNA and small amounts of biological material (Ladoukakis & Zouros, 2017). Mitochondrial DNA lacks recombination and infrequently shows gene rearrangements making it ideal for population genetic studies as well (Chen et al., 2019). Additionally, molecular species identification assists in defining species of insects that are cryptic, sexually dimorphic, polyphenic and have various developmental stages in their life cycle (Funk & Omland, 2003).

The 13 protein coding regions are therefore used in evolutionary studied in insect families, genera, and species (Mandal et al., 2014). Protein coding genes are divided into three categories ("good", "medium", and "poor" performers) in recovering phylogenetic trees among distant relatives (Ptaszyńska et al., 2012), these categories are based on tests performed by Zardoya & Meyer (1996). Genes in the "good" category are ND2, ND4, ND5, COI and CYTB, while COII, COIII, ND1 and ND6 are classed in "medium" category and ATP6, ATP8, ND3 and ND4L in the "poor" category (Zardoya & Meyer, 1996).

The small ribosomal subunit, 12s rRNA is used for genetic diversity studies in many phyla due to it being highly conserved, while the large ribosomal subunit (16s rRNA) is useful in differentiation of families, subfamilies and tribes, of closely related species or populations. The 16s rRNA is therefore useful in resolving monophyly issues in phylogenetic reconstructions. Mitochondrial protein coding genes have a higher evolution rate than rRNA genes (Mandal et al., 2014).

In mitochondrial DNA of insects, the AT-rich region (control region) is generally located between 12s rRNA and $tRNA^{Met}$ and contains the signals for transcription and translation (McDonagh et al., 2016). The AT-region is a less popular molecular marker used in phylogenetic studies of insects due to high AT-content and presence of repeats, including homopolymeric stretches. The AT-region is not a frequently used marker in phylogenetic studies of insects as universal primer design is problematic due to possible tRNA transposition in different insects, therefore primer working for one taxon might not work for another (Roy, 2014).

Gaining a more comprehensive insight into biodiversity is the driving force behind identifying species using molecular identification methods (Nneji et al., 2020). The most popular genetic marker used for the identification of insects' species is a 700 bp segment located in the cytochrome oxidase I (COI) gene (DNA barcode), as it is a highly conserved region with high interspecific variation and low intraspecific variation (Hebert et al., 2003; Ratnasingham & Hebert, 2007). Genetic distance calculations assistant in interpreting sequence similarities between DNA barcodes, based on the assumption that interspecific genetic variations surpass intraspecific genetic variation (Meiklejohn et al. 2019). DNA barcoding increases the rate of species documentation as this method can identify potential species much faster than conventional taxonomy(Dincă et al., 2011). Currently, rapid species identification and biodiversity assessments are particularly important (Ziaja et al., 2016). Humans have caused a drastic decrease in

biodiversity worldwide, especially in species that are economically and nutritionally valuable, like Saturniidae (Silva-Brandão et al., 2009). It is therefore important to identify and conserve biodiversity before it is lost.

Although promising, DNA barcoding has some limitation and pitfalls that must be considered. The maternal inheritance of mtDNA is both an advantage and limitation (Meiklejohn et al., 2019). Phenomena such as hybridization and endosymbionts cause the transfer of mitochondrial genes outside of the species and may generate misleading results (Lis et al., 2016; Nneji et al., 2020). The possible presence of nuclear mitochondrial DNA (NUMTs) are a major pitfall often underestimated when barcoding (Anderson & Leite, 2012). NUMTs or pseudogenes are copies of non-functional mtDNA in the nuclear genome (Meiklejohn et al., 2019; Pentinsaar et al., 2020). When amplifying target DNA, PCR primers can also coamplify NUMTs. Pseudogenes do not experience selective pressure therefore accumulate mutations at a higher rate than coding regions, resulting in nucleotide substitutions or frameshifts which produce premature stop codons in coding regions (Cameron, 2014: Anderson & Leite, 2012). In some cases, NUMTs can be identified by translating sequences into amino-acids and checking for premature stop codons. The accidental amplification of NUMTs may introduce sequence ambiguities and inflate estimates of the number of species (Song et al., 2008). This issue therefore cannot be ignored, and additional markers should be also analyzed in addition to the COI gene. COI has low level of polymorphism thus provides inadequate resolution for intraspecific and intrapopulation studies (Mandal et al., 2014). Different genes and lineages also have varying mutation rates and single genetic markers generally provide insufficient resolution in intrapopulation studies (Hubert & Hanner, 2016). Therefore, selection of mitochondrial markers should be based on comparisons of the complete mitochondrial genomes of conspecific individuals from distinct populations across their distribution range (Kvie et al., 2016). It has been established that optimal mitochondrial marker selection for intraspecific studies is crucial for optimal resolution. Optimal mitochondrial markers for intrapopulation studies possess the following characteristics: conserved regions to which species-specific PCR primers can bind to, high levels of polymorphism, PCR amplicons should be located within PCGs to allow for the potential detection of NUMTs (Hazkani-Covo et al., 2010; Mandal et al., 2014).

Together with DNA barcodes it is important to keep records and additional supporting information associated with the newly identified specimen (Meiklejohn et al., 2019). This is of particular importance as there are currently few safeguards against misidentified species that are uploaded on public databases like BOLD (Rulik et al., 2017). Therefore, to make the most accurate species identification it is advised to combine traditional taxonomy with DNA barcoding (Packer et al., 2009).

1.10 Aims and objectives

This study aimed to expand the available mitogenomic data for African Saturniidae as well as preliminarily assess the genetic diversity of the most exploited edible African Saturniidae *Gonimbrasia belina*. The following objectives were set out to achieve this aim:

1. To generate mitogenomic data for seven species in three tribes: *H. dyops, G. tyrrhea, B. alcinoe, N. cytherea* (Bunaeini), *E. bauhinia* (Attacini), *V. ducalis* and *V. grimmia* (Micragonini), and infer their phylogenetic placement within the family Saturniidae

2. To investigate the genetic diversity, population structure and phylogeography of *Gonimbrasia belina* in South Africa, Namibia and Botswana.

Chapter 2: Mitogenomics and phylogeny of seven African Saturniidae (Lepidoptera)

2.1 Abstract

African Emperor moths (Lepidoptera: Saturniidae) have been understudied compared to their Asian counterparts and mitochondrial phylogenies of the family have so far included only *Gynanisa maja* and *Gonimbrasia belina*, the edible caterpillars most utilized in southern Africa. I attempted to fill this gap by sequencing the complete mitochondrial genomes of seven African species (*Epiphora bauhinia, Heniocha dyops, Gonimbrasia tyrrhea, Bunaea alcinoe, Nudaurelia cytherea, Vegetia ducalis* and *Vegetia grimmia*), and performed comparative mitogenomic and phylogenetic analyses using other mitogenomes publicly available for the family. The nucleotide composition for all seven mitogenomes is highly biased towards A+T (average 82.30%), as typically is the case for insects, and the average AT-skew is slightly negative (-0.12). ATN codons initiate all protein coding regions (PCGS) except for COI (CGA), a common feature in Saturniidae. The tribe Micragonini (represented by *Vegetia ducalis* and *Vegetia grimmia*) is here included for the first time in the mitochondrial phylogeny of Saturniidae. Bayesian Inference and Maximum likelihood phylogenetic reconstruction was performed separately for three datasets: (1) PCG123 - all PCGs and all codon positions, (2) PCG12 - all PCGs with 3rd codon position removed; and (3) PCG123+rRNA – all PCGs and two rRNAs (16s rRNA and 12s rRNA). Previous studies showed similar results, in that all phylogenies failed to recover the monophyly of Saturniini. However, Attacini, Bunaeini and Micragonini were recovered as monophyletic groups. The inclusion of the rRNA genes did not result in relevant differences.

Keywords: Phylogenetics, Emperor moths, mitogenomics, Saturniinae

2.2 Introduction

Lepidoptera has over 180,000 species described globally making it the fourth most diverse insect order (Goldstein, 2017; Nneji et al., 2020). Some lepidopteran species are of great economic importance: for example, species like *Antheraea assamensis* and *Samia cynthia* (Saturniidae) are farmed for silk production, and over 400 other species are utilized by humans for food or medicine, particularly at the caterpillar stage (Yen, 2015). Most African Emperor moths, except *Argema mimosae* (Saturniini) and *Epiphora bauhiniae* (Attacini) have lost the ability to spin silk (Ghazoul, 2006). Therefore, *A. mimosae* and *E. bauhiniae* (Attacini) have the potential to be the foundation of sericultural practice on the African continent, yet information on African silk producing species is scarce (Kim et al., 2016; Liu et al., 2020). Edible insects are regarded as a naturally renewable protein source (van Huis, 2016). The importance of edible Lepidoptera is often overlooked by western and westernized societies, yet they are not only an important nutritional resource but an economic resource providing seasonal income for many communities globally during times of hardship, including large regions of southern Africa (Makhado et al., 2014). The most biodiverse continent in the world for edible insects is Africa and entomophagy is widespread in about 36 countries, some of which consume large quantities of caterpillars of Saturniidae species (Ramos, 2005).

The family Saturniidae contains 3,454 species distributed worldwide in various diverse terrestrial habitats (Kitching et al., 2018). Bunaeini, Attacini, Saturniini, Micragonini, Eochroini and Urotini are the six African Saturniidae tribes which contain 50 genera and an estimate of 400 species (Oberprieler, Morton & van Noort, 2021). Yet, taxonomic inconsistencies and instability in the Saturniidae family has resulted in difficulty determining the exact number of species (Kitching et al., 2018; Hurst et al., 2020). The error of allocating specimens the wrong species name, results in the number of Saturniidae species occurring and consumed in Africa being overstated or understated, which in turn impacts the implementation of conservation strategies (Hurst et al., 2020).

A minimum of 50 African Saturniidae species larvae are consumed throughout the continent (Hlongwane, Slotow and Munyai, 2020). Majority of edible Saturniidae species are found in three tribes, Bunaeini, Micragonini and Urotini, and the following genera: *Athletes, Bunaea, Bunaeoides, Bunaeopsis, Cinabra, Cirina, Gonimbrasia, Gynanisa, Heniocha, Imbrasia, Lobobunaea, Melanocera, Nudaurelia, Pseudobunaea*, *Rohaniella* (Bunaeini), *Goodia, Micragone* (Micragonini), *Pseudaphelia, Urota* and *Usta* (Urotini) (Silow ,1976; Huelsenbeck et al., 2013; Thomas, 2013; Hlongwane, Slotow & Munyai, 2020). African Emperor moths are understudied despite their ecological and economic relevance, as the caterpillars of many species are utilized for human consumption (van Huis, 2016). Mopane worms, vernacular for the caterpillars of *Gonimbrasia belina* (Bunaeini) and other Saturniidae in South Africa, are widely harvested in southern Africa due to their high nutrient content (Illgner et al., 2016).

Mopane worm populations periodically erupt in mopane tree (*Colophospermum mopane*) dominant vegetations (Thomas, 2013; Hlongwane, Slotow & Munyai, 2020). These caterpillars are a valuable protein source and have become a desirable economic commodity for commercial harvesters. It is estimated that 9.5 billion mopane worms are harvested and traded per annum in southern Africa (Halloran et al., 2015). Increased demands for favoured edible Saturniidae species, habitat destruction, unregulated harvesting methods, lack of population distribution data and monitoring are placing wild populations in serious danger of decline (Thomas, 2013). Despite the importance of caterpillar consumption on the African continent, there are no designated protected areas for conservation of edible Saturniidae or any other edible insects in southern Africa (Dzerefos, 2018).

Mitogenomic data is commonly used in insect phylogenetic reconstructions (Dong et al., 2016). Additionally, current phylogenetic methodological improvements have made the recovery of relationships between taxa affected by accelerated evolutionary rates and compositional heterogeneity possible (Song et al., 2008). Asian species have been the main focus of mitochondrial phylogenies of Saturniidae (Dong et al., 2016; Jiang et al., 2009; Kim et al., 2018; Yang et al., 2016; Zhu et al., 2020). African Saturniidae have remained largely unsurveyed, and mitochondrial phylogenies of the family have so far included only *G. belina* and *Gynanisa maja* (Bunaeini), two of the most exploited edible caterpillars in southern Africa (Langley et al., 2020). Currently, most research on the genetic diversity of Saturniidae has focused on species farmed in Asia for silk production (Regier et al., 2008). The limited availability of baseline genetic information on African Saturniidae species has impeded assessments of phylogenetic relationships, phylogeographic structure and genetic diversity of these species.

In an attempt to rectify this knowledge gap in African Saturniidae, the aim of this study was to generate baseline mitogenomic data and to assess the phylogenetic position of seven species in three tribes: *Heniocha dyops, Gonimbrasia tyrrhea, Bunaea alcinoe, Nudaurelia cytherea* (Bunaeini), *E. bauhinia* (Attacini), and *Vegetia ducalis* and *Vegetia grimmia* (Micragonini). For that purpose, I sequenced and described the complete mitogenome of one individual of each species and inferred their phylogenetic relationships within the family.

2.3 Materials and methods

2.3.1 Sample collection and DNA extraction

Caterpillars and adult specimens of representative of the species *E. bauhinia, H. dyops, G. tyrrhea, B. alcinoe, N. cytherea, V. ducalis* and *V. grimmia* were collected at various locations in Namibia and South Africa between 15 March 2018 and 12 March 2020 (**Table 2.1**). Specimens were euthanized by freezing within few hours of collection from the field, whenever possible. Adult specimens were stored in sealed petri dishes at -20℃, and caterpillars were stored in 100%

ethanol at room temperature until DNA extraction. DNA from the legs of caterpillars and adult specimens was extracted using a standard phenol- chloroform protocol (Barnett & Larson, 2012).

Table 2.1 Sample list. Collection data for seven species of African Saturniidae used for the recovery of the complete mitogenomes, comparative mitogenomics and phylogenetic reconstruction within the family.

Species	Sample	Collection date	Country	Region	GPS Co-ordinates
Bunaea alcinoe (Stoll, 1780)	BA1.6	11 October 2019	South Africa	Gauteng	−25.7479, 28.2293
Epiphora bauhinia (Guérin-Méneville, 1832)	GM5.2	26 February 2019	Namibia	Okonjima	−20.7579, 16.7898
Gonimbrasia tyrrhea (Guérin-Méneville, 1832)	GT1.1	25 May 2019	South Africa	Western Cape	−32.7681, 18.1607
Heniocha dyops (Maassen, 1872)	GM8.2	15 March 2018	Namibia	Windhoek	−22.8577, 17.1089
Nudaurelia cytherea (Fabricius, 1775)	NC1.2	12 October 2019	South Africa	Western Cape	−34.3322, 18.9878
Vegetia ducalis (Jordan, 1922)	VD1	22 May 2019	South Africa	Western Cape	−33.4201, 18.4005
Vegetia grimmia (Geyer, 1831)	VG2	12 March 2020	South Africa	Western Cape	−34.3053, 18.4619

2.3.2 DNA barcoding and morphological Identification

Morphological identification of the specimens was performed by Rolf Oberprieler (CSIRO, Australia) following the available literature and taxonomic keys (**Figure 2.1**) (Pinhey, 1972; Cooper, 2002). To ensure that species were accurately identified prior to recovery of the mitogenomes, a representative specimen from each species were sequenced for the standard COI barcoding region (700 bp), using combinations of various polymerase chain reaction (PCR) primer pairs. The primer pair LepF/LepR was used for most species as they are modifications of the “universal Folmer primers” (LCO1490 and HCO2198) specifically designed to amplify the barcoding region of Lepidoptera (**Table 2.2**) (Foottit et al., 2008). All PCR amplifications were performed in a total volume of 5 µL, containing 2x (2.5 µL) of Qiagen Multiplex PCR Kit (QIAGEN), 0.5 µL of each primer (0.5 µM), 0.5 µL of Milli-Q water and 1 µL (~100 ng/ µL) of template DNA. Thermocycling conditions consisted of initial denaturation at 95°C for 15 min; 35 cycles of 94°C for 30 s, 50°C for 90 s and a final extension at 72°C for 10 min. PCR products which presented clear bands in a

1.5% agarose gel were sequenced unidirectionally using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), at the Central Analytical Facilities of Stellenbosch University. Sequences were manually edited and trimmed on Geneious Prime v2020.2.2 (https://www.geneious.com/) and aligned using MAFFT algorithm plug-in (Katoh & Standley, 2013) on Geneious Prime. The DNA barcodes produced were compared to all publicly available COI sequences for each genus (*Bunaea, Gonimbrasia, Vegetia, Nudaurelia, Epiphora* and *Gynanisa*) on the Barcode of Life Database (BOLD Systems; https://v3.boldsystems.org/) and on the National Center for Biotechnology Information (NCBI; GenBank).

Table 2.2 Primer pairs. Primers and annealing temperatures used for PCR amplification. *Primer used for unidirectional Sanger sequencing of the standard barcoding COI region.

Species	Barcoding primers	Sequence	Ta (°C)
Bunaea alcinoe	Bar-Bel-F	ATTCTACTAATCATAAAGATATTGG	54
	Lep-R*	TAAACTTCTGGATGTCCAAAAAATCA	
Epiphora bauhinia	Lep-F	ATTCAACCAATCATAAAGATATTGG	52
	Lep-R*	TAAACTTCTGGATGTCCAAAAAATCA	
Gonimbrasia belina	Bar-Bel-F	ATTCTACTAATCATAAAGATATTGG	54
	Bar-Bel-R*	ACTTCTGGGTGTCCAAAAAATC	
Gonimbrasia tyrrhea	Lep-F	ATTCAACCAATCATAAAGATATTGG	52
	Lep-R*	TAAACTTCTGGATGTCCAAAAAATCA	
Gynanisa maja	Lep-F	ATTCAACCAATCATAAAGATATTGG	52
	Lep-R*	TAAACTTCTGGATGTCCAAAAAATCA	
Heniocha dyops	Lep-F	ATTCAACCAATCATAAAGATATTGG	52
	Lep-R*	TAAACTTCTGGATGTCCAAAAAATCA	
Nudaurelia cytherea	Lep-F	ATTCAACCAATCATAAAGATATTGG	52
	Lep-R*	TAAACTTCTGGATGTCCAAAAAATCA	
Vegetia ducalis	Lep-F	ATTCAACCAATCATAAAGATATTGG	52
	Bar-Bel-R*	ACTTCTGGGTGTCCAAAAAATC	
Vegetia grimmia	Lep-F	ATTCAACCAATCATAAAGATATTGG	52
	Lep-R*	TAAACTTCTGGATGTCCAAAAAATCA	

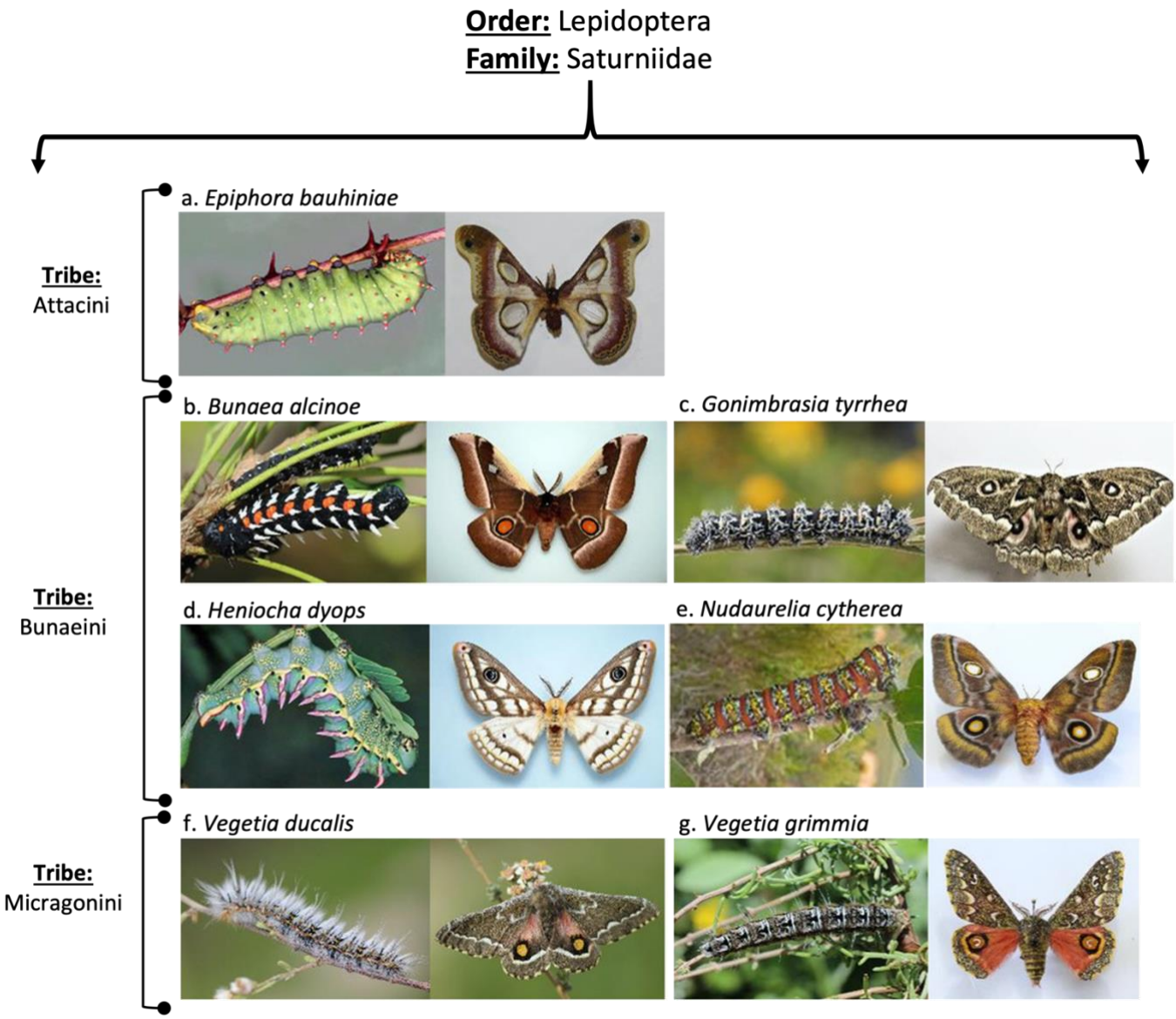

Figure 2.1 Representative specimens of seven African Saturniidae species. Larval and adult specimens used for phylogenetic reconstruction and mitogenome sequencing in this study. (a) *Epiphora bauhinia*, (b) *Bunaea alcinoe*, (c) *Gonimbrasia tyrrhea*, (d) *Heniocha dyops*, (e) *Nudaurelia cytherea*, (f) *Vegetia ducalis*, and (g) *Vegetia grimmia*.

2.3.3 High-throughput sequencing for recovery of complete mitochondrial genomes

DNA from one representative of each species was individually sequenced using the Ion Torrent Proton™ sequencing platform (ThermoFisher Scientific) available at the Central Analytical Facility of Stellenbosch University, South Africa. In brief, libraries were prepared using Ion Express™ and gDNA Fragment Library (ThermoFisher Scientific, Waltham, MA, USA) according to the MAN0009847 REV J.0 protocol. The templates were prepared by diluting libraries to a concentration of 60 pM. The diluted libraries were combined in equimolar amounts for sequencing

preparation using Ion 540™ Chef (ThermoFisher Scientific). Twenty-five microliters of diluted, pooled libraries were loaded onto the Ion Chef liquid handler for template preparation and enrichment using Ion 540™ Chef reagents, solutions and supplies according to protocol MAN0010851 REVF.0. Enriched ion sphere particles were loaded onto Ion540™ Chip. Massive parallel sequencing was performed on Ion Torrent S5™ system using sequencing solutions, reagents and supplies according to protocol MAN0010851 REVF.0. Flow space calibration and base caller analysis was performed using standard analysis parameters in the Torrent Suite Version 5.12 Software.

2.3.4 Mitogenome mapping, assembly, and annotations

Reference-based assemblies of the HTS reads were performed using complete mitogenomes available on GenBank. The closest relative for each species was selected based on COI sequence similarity estimated from DNA barcodes. The reads for *B. alcinoe, G. tyrrhea* and *N. cytherea* were mapped against the reference mitogenome of *G. belina* (NC_046032), *H. dyops* was mapped against of *Gy. maja* (NC_046033)*, E. bauhinia* was mapped against *Attacus atlas* (NC_021770), and *V. ducalis* and *V. grimmia* were mapped against *Actias luna* (NC_045899). Mapping and assembly of the NGS reads was performed on the Geneious Prime with low to medium sensitivity option and fine tuning up to five iterations. Open reading frames for the typical 13 metazoan mitochondrial protein coding genes (PCGs) were identified on Geneious Prime using the invertebrate mitochondrial genetic code. The twenty-two transfer RNA (tRNA) genes were identified with ARWEN software (http://130.235.244.92/ARWEN/) using the default composite metazoan mitochondrial genetic code. The two ribosomal RNA (rRNA) genes and the AT-rich region were annotated by comparison to other complete Saturniidae mitogenomes available on GenBank.

2.3.5 Comparative mitogenomics

Nucleotide composition and AT- and GC-skews were calculated using Geneious Prime, according to the following formula: AT-skew = (A - T) / (A + T) and GC-skew = (G – C) / (G + C). Gene overlapping regions and intergenic spaces (IGS) were counted manually.

2.3.6 Phylogenetic analyses

The phylogenetic reconstruction of the family Saturniidae included the seven new mitogenomes and all other sequences publicly available as of November 2020 (**Table S2**). The final dataset included 36 sequences across 25 species in four tribes Saturniini (n= 20), Attacini (n= 6), Bunaenini (n= 8) and Micragonini (n= 2). *Bombyx mori* (NC_002355) and *Bombyx mandarina* (NC_003395) (Lepidoptera: Bombycidae) were used as outgroups. The mitogenomes retrieved from GenBank were manually curated for apparent sequencing and annotations errors (**Table 2.3**). Small adjustments were made to the mitogenomes of *Neoris haraldi* (NC_036765), *Samia cynthia*

ricini (JF961379), *Saturnia jonasii* (MF346379) and *B. mori* (NC_002355), mostly involving errors causing frameshift alterations in conserved PCGs such as COI, COIII and ND4 due to artefactual indels that had not been curated in those publicly available sequences. *Samia cynthia ricini* had premature stop codons in the reading frame of COI and COIII resulting in significantly shorter genes. *Neoris haraldi* had a 2-bp deletion near the 3'-end of ND3 causing a shift in the reading frame and shortening of the gene. *Bombyx mori* had an insertion of 1 bp in ND4 causing a shift in the reading frame of the gene.

Table 2.3 Curated mitogenomes. Species used in the mitochondrial phylogenetic reconstruction of Saturniidae in which sequencing errors have been identified and corrected.

Gene	Species	GenBank	Changes made	Result
COI	*Samia cynthia ricini*	JF961379	Deleted G between 2,298 and 2,299 bp	Gene was extended to the correct length (1,536 bp)
COIII	*Samia cynthia ricini*	JF961379	Deleted G between 4,910 and 4,911 bp	Gene was extended to the correct length (789 bp)
ND3	*Neoris haraldi*	NC036765	Added TA at 5,899 - 5,900	Reading frame was restored
ND4	*Bombyx mori*	NC002355	Deleted A between 3,106 and 3,107 bp	Reading frame was restored
ND5	*Samia cynthia ricini*	JF961379	Deleted A between 7,039 and 7,040 bp	Gene was extended to the correct length (1,746 bp)
ND5	*Neoris haraldi*	NC036765	Deleted ATAT between 6,431 and 6,432 bp	Reading frame was restored

Phylogenetic analyses were performed separately on three datasets: (1) PCG123 - all PCGs and all codon positions, (2) PCG12 - all PCGs with 3rd codon position removed; and (3) PCG123+rRNA – all PCGs and rRNAs (16s rRNA and 12s rRNA). PCGs were extracted from each individual sequence and stop codons were removed for all datasets. Translation alignments were performed separately for each PCG using the MAFFT algorithm in Geneious Prime. For PCG123, the 13 individual PCG alignments were concatenated to form a single alignment. The PCG12 concatenated alignment was created by masking the 3rd codon of PCG123 concatenated alignment by selecting the 3rd codon position under the default settings in Geneious Prime. The PCG123+rRNA had an additional step that included extracting the two ribosomal RNA genes (12s rRNA and 16s rRNA) together with the PCGs. The translation alignment was performed separately for each PCG (without stop codon), and a nucleotide alignment was performed for the rRNAs. The two rRNAs alignments together with the 13 PCG alignments were concatenated to create single PCG123+rRNAs alignment. The concatenated alignments for the three datasets were individually edited using GBlocks v0.91 (https://ngphylogeny.fr/tools/tool/276/form) for removing gaps and poorly aligned regions.

The final alignments for the three datasets were partitioned using PartitionFinder2 on XSEDE (Lanfear et al., 2016) on the CIPRES Portal ver. 3.3 (Miller et al., 2010) (https://www.phylo.org/) to determine the best-fitting partitioning schemes and evolutionary model for the construction of phylogenetic trees. Partitioning was performed using default settings for DNA for maximum of 30 hours and GTR+I+G was considered the best model for all datasets. Bayesian Inference (BI) analyses for all datasets was performed under the GTR+I+G nucleotide substitution model selected with PartitionFinder using the software MrBayes ver.3.2.6 (Huelsenbeck & Ronquist, 2001), also available on CIPRES. BI was conducted as follows: two independent runs of four heat chains, ten million generations run simultaneously, resampling ever 1,000 generations, first 25% of trees discarded as burn-in and the decision criterion for the convergence of the two runs set as an average split frequency of ≤ 0.01. Confidence values of all three BI trees were calculated using Bayesian posterior probabilities (BPP). Maximum likelihood (ML) trees were constructed for all three datasets in IQ-Tree under the GTR+F+I+G4 substitution model (Minh et al., 2020). The best evolutionary model was determined using automatic model selection available in IQ-Tree (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017). To determine branch support, we used both ultrafast bootstrapping and approximate likelihood ratio (SH-aLRT) branch test at 1,000 replicates (Hoang et al., 2017). All trees were drawn using FigTree v1.4.3. (http://bio.tools/Figtree).

2.4 Results and discussion

2.4.1 Comparative mitogenomics of Saturniidae

2.4.1.1 Genome organization

Ion Torrent generated HTS reads varying between 11 million (*V. ducalis*) and 21 million (*E. bauhiniae*), with an average size of 182 bp. The total number of mapped reads ranged from 33,793 (*N. cytherea*) to 191,925 (*H. dyops*). Average sequence coverage varied between 349× (*N. cytherea*) and 1810× (*H. dyops*) (**Table 2.4**). The size of the complete mitogenomes ranges from 15,218 bp (*V. ducalis*) to 15,363 bp (*N. cytherea*), in line with those of other Saturniidae (Kim et al., 2018; Langley et al., 2020). The complete mitogenomes of the seven newly sequenced African Saturniidae species are identical in gene content and organization, with 13 PCGs, 22 transfer RNA genes (tRNAs), two rRNAs and an AT-rich non-coding region. Twenty-three genes (nine PCGs and 14 tRNAs) are located on the major strand (J) and four PCGs, eight tRNAs and two rRNAs were located on the minor strand (N) (**Table S1**). The non-coding AT-rich region was located between 12s rRNA and $tRNA^{Met}$ and ranged from 337 to 377 bp. The mitogenomes of Saturniidae gene order is conserved across all species and a single difference, the translocation of $tRNA^{Met}$ and $tRNA^{Gln}$ relative to $tRNA^{Ile}$ (M-I-Q) was observed when compared to the hypothetical ancestral Arthropoda (I-Q-M) (Boore, 1999) (**Figure 2.2**). The new mitogenomes are biased towards A and T

with the A+T content ranging from 79.1% in *H. dyops* to 80.5% in *N. cytherea* and *V. ducalis* (**Table 2.5**), as typically is the case in insects (Mandal et al., 2014).

Table 2.4 High-throughput sequencing (HTS) and assembly. Samples and results for the mapping and assembly of HTS reads for the recovery of the complete mitochondrial genomes of seven African Saturniidae (Lepidoptera).

Sample	Number of reads	Average read length (bp)	Number of mapped reads	Coverage	Consensus sequence length (bp)
Bunaea alcinoe BA1.6	15,634,521	187	49,550	582x	15,305
Epiphora bauhinia GM5.2	21,284,325	173	108,486	1147x	15,259
Gonimbrasia tyrrhea GT1.1	17,135,076	177	132,321	1412x	15,299
Heniocha dyops GM8.2	15,407,906	173	191,295	1810x	15,306
Nudaurelia cytherea GM5.2	19,034,516	174	33,793	349x	15,363
Vegetia ducalis VD1	11,321,643	195	42,382	509x	15,218
Vegetia grimmia VG2	14,153,225	193	88,880	1048x	15,253

Table 2.5 AT- and GC-skew. AT and GC content as well as AT-skews and GC-skews of the complete mitochondrial genome of seven African Saturniidae species.

Species	A%	C%	G%	T%	A+T%	G+C%	AT-skew	GC-skew	Size (bp)
Bunaea alcinoe	40.20	12.40	6.90	40.20	80.40	19.30	-0.03	-0.29	15,305
Epiphora bauhinia	39.50	12.00	7.90	40.50	80.00	19.90	-0.01	-0.21	15,259
Gonimbrasia tyrrhea	39.60	12.30	7.30	40.70	80.30	19.60	-0.01	-0.26	15,299
Heniocha dyops	38.60	12.90	7.80	40.50	79.10	20.80	-0.02	-0.25	15,306
Nudaurelia cytherea	39.00	11.50	7.80	41.50	80.50	19.30	-0.01	0.20	15,363
Vegetia ducallis	39.00	11.50	7.80	41.50	80.50	19.30	-0.01	0.20	15,218
Vegetia grimmia	38.90	11.70	7.80	41.40	80.30	19.50	-0.01	0.20	15,253

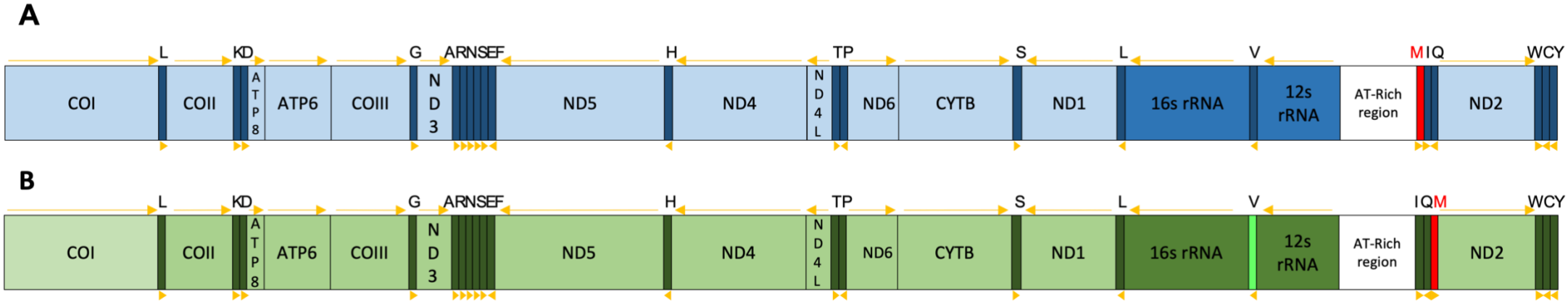

Figure 2.2 Linear map of the complete mitogenome. Linear map of the complete mitochondrial genomes of *Gonimbrasia tyrrhea, Epiphora bauhinia*, *Heniocha dyops*, *Bunaea alcinoe*, *Vegetia ducalis* and *Vegetia grimmia* (Lepidoptera: Saturniidae). (A) African Saturniidae, and (B) Hypothetical ancestral insect mitochondrial genome.

2.4.1.2 Mitochondrial protein-coding genes

PCGs represent 73% of the total length of mitogenomes, with A+T content ranging from 77.9% to 79.8%. The gene with highest average A+T content across all species is ATP8 (91.00%) and lowest is seen in COI (71.70%) (**Figure 2.3**), consistent with previous studies on Saturniidae (Jiang et al., 2009; Singh et al., 2017). The AT-skew of the major strand is negative for all genes except ND1, ND4, ND4L and ND5, which all have a slightly positive AT-skew in all species. Additionally, all PCGs for all species in this study have a slightly negative GC-skew (**Figure 2.3**).

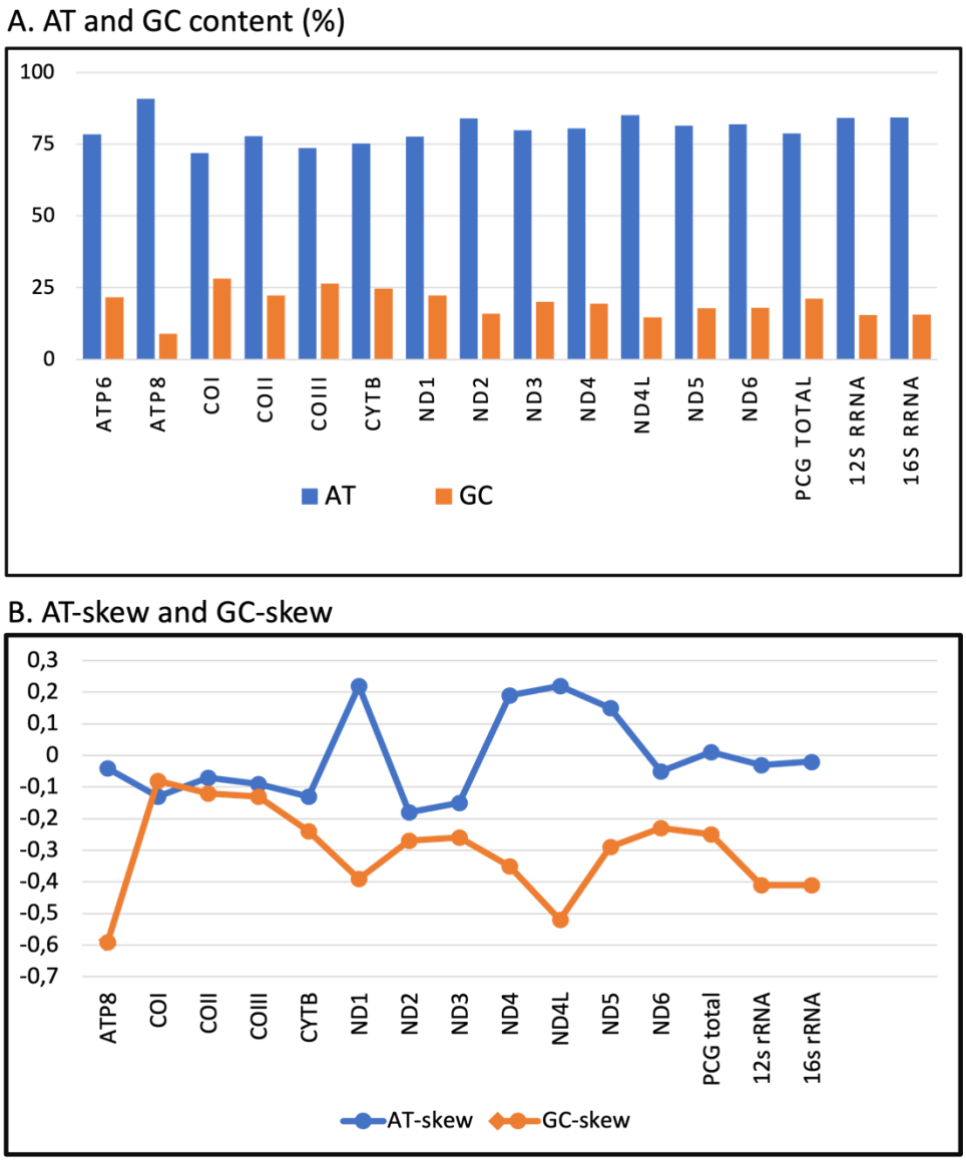

Figure 2.3 Nucleotide composition of Saturniidae mitogenomes. Seven complete mitochondrial genomes of African Saturniidae (Lepidoptera) average nucleotide composition. (A) AT% and GC% content and (B) AT- skew and GC- skew for each of the 13 protein-coding genes (PCG), total for PCG and two rRNAs (12s rRNA and 16s rRNA).

ATN start codons (ATA, ATT, ATG and ATC) initiated all PCGs, except for COI which was initiated by CGA (**Figure 2.4**). The CGA start codon for COI has been reported as highly conserved in Lepidoptera (Bao et al., 2019). GTG as a start codon for COII was not observed in this study but it has been reported in *Antheraea assamensis*, *Eryogina pyretorum*, *Samia boisduvali* and *Samia ricini* (Jiang et al., 2009; Kim et al., 2012; Singh et al., 2017). The majority of the PCGs (9/13) terminates with the complete TAA or TAG stop codon while four (COI, COII, ND3 and ND5) terminate with incomplete T or TA stop codons (**Figure 2.5**). All the mitogenomes in this study follow the trend of other Saturniidae with the most frequently used codons being UUA (Leu), AUU (Ile), UUU (Phe) and UCA (Ser). These codons have a high A/T content rather than G/C, and this reflects the high A/T composition in the insect mitochondrial genome (Bao et al., 2019).

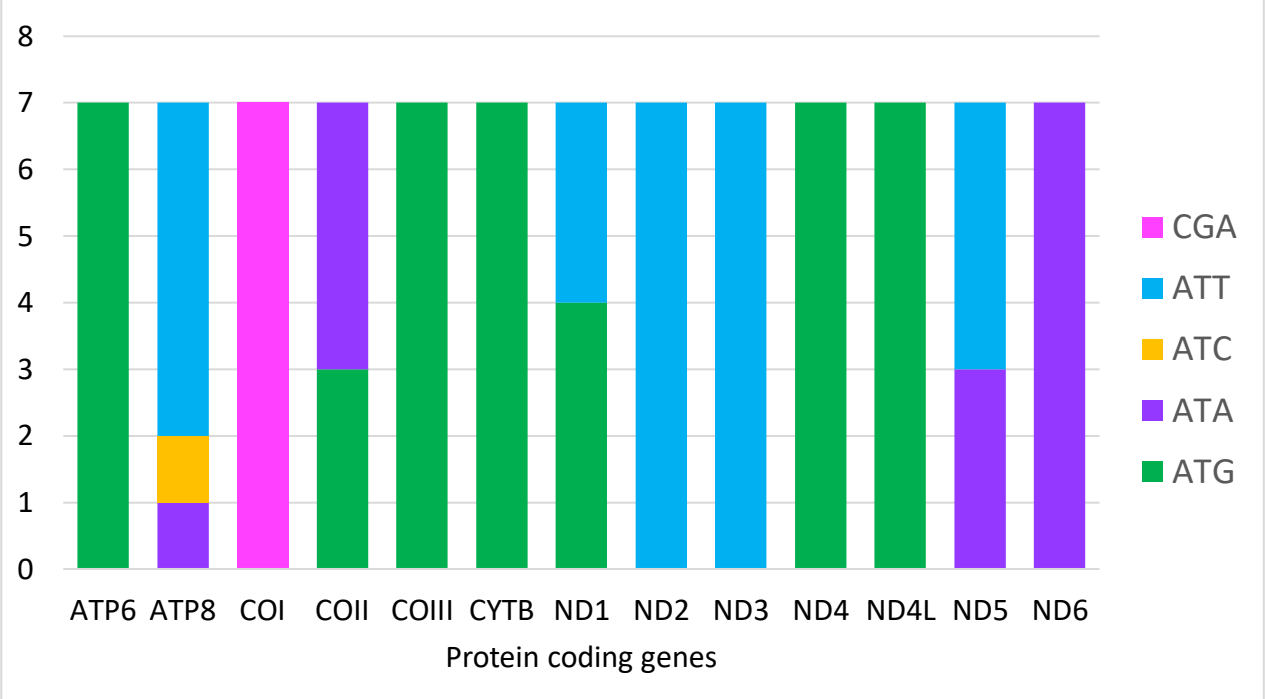

Figure 2.4 Usage of start codons in Saturniidae mitogenomes. The utilization of ATN and CGA start codons in 13 protein coding genes of seven African Saturniidae species.

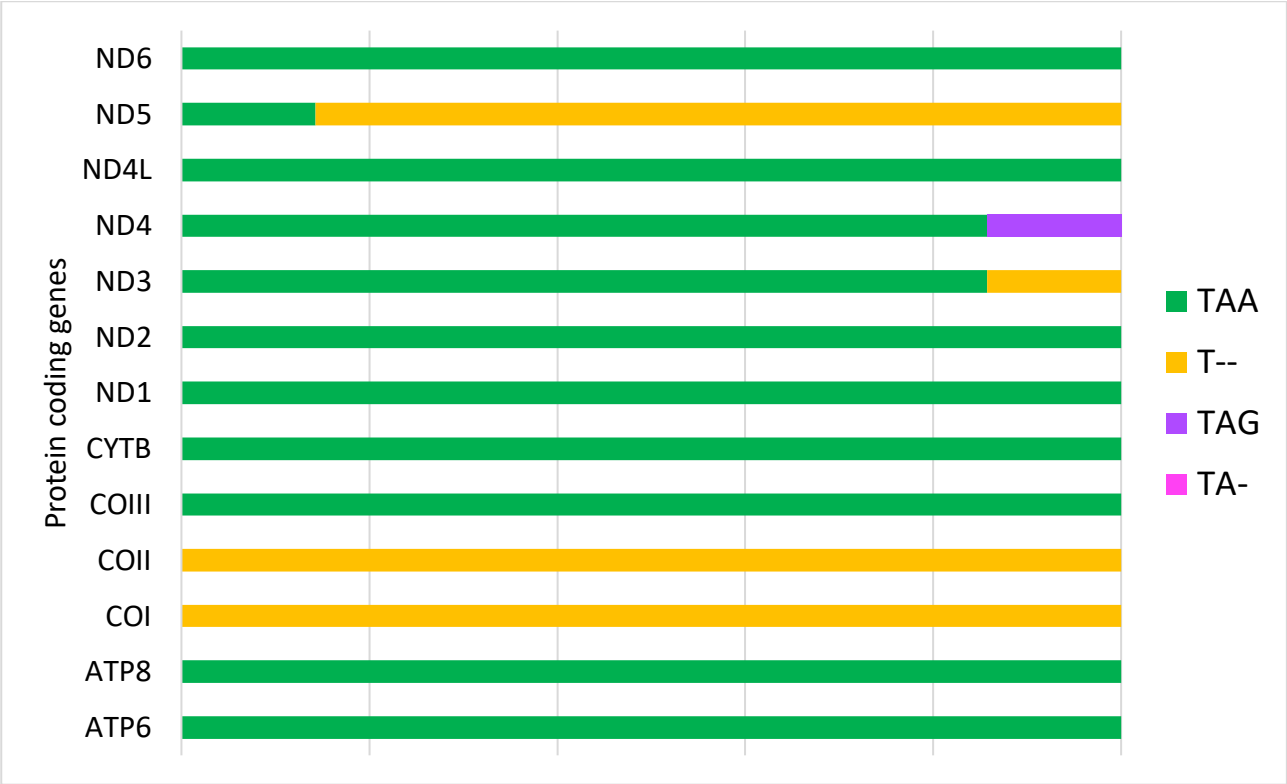

Figure 2.5 Stop Codons. The utilization of stop codons in 13 protein coding genes in seven mitogenomes of seven African Saturniidae species in this study.

2.4.1.3 Intergenic spacer regions and overlapping sequences

The mitogenomes are compact with intergenic spaces (IGS) varying from 1 to 50 bp in length. The largest IGS is between ND2 and $tRNA^{Gln}$ (47-59 bp) for all mitogenomes, but *B. alcinoe* was the exception with the largest IGS located between ND1 and $tRNA^{ser2}$ (55 bp). Gene overlapping regions in the seven mitogenomes range from 3 to 53 bp in length. The longest overlap is between $tRNA^{cys}$ and $tRNA^{trp}$ (averaging 9 bp), similar overlap between ATP6 and ATP8 genes (averaging 7 bp), the same trend is observed in all seven mitogenomes (**Table S1**).

2.4.2 Phylogenetic position of African Saturniidae within the family

Mitochondrial genomes are important genomic resources for reconstructing phylogenetic relationships among species (Cameron, 2014). Until the present study, the only African Saturniidae mitochondrial genomes available on GenBank were those of *G. belina* and *Gy. maja*, and the family has been mostly represented by Asian species (Langley et al., 2020). Our phylogenetic reconstructions (BI and ML) included all mitogenomes currently available for the family, as well as the seven additional sequences generated in this study (**Figure 2.6**; **Figure 2.7**; **Figure S 1**; **Figure S 2**). The ML and MrBayes trees had the same tree topologies, however MrBayes was able to recover higher statistical support for deeper nodes (**Figure S 1**; **Figure S 2**). Different inference models (ML and BI) yielded inconsistent results for the same data, especially with regards to the order of deeper nodes (Yuan et al., 2016). A study on the mitochondrial phylogeny of Coleoptera

showed that different phylogenetic reconstructions methods impacted tree topologies (Yuan et al., 2016). Although tree topologies in this study remained the same, several nodes in the ML trees had lower statistical support. I therefore will focus on discussing the topologies of phylogenetic trees constructed using BI method.

The PCG123 tree and PCG12 tree (**Figure 2.6**; **Figure S 1**) recovered identical topologies, and *H. dyops, B. alcinoe, N. cytherea*, and *G. tyrrhea* were positioned in the monophyletic clade Bunaeini, with strong statistical support (BPP = 1). These species together with *G. belina* and *Gy. maja* form part of the tribe Bunaeini which contains most of the edible African Saturniidae species and is regarded as the most relevant taxonomic tribe in the context of entomophagy. The mitogenomes of *V. ducalis* and *V. grimmia* are the first available for the tribe Micragonini, which was recovered as a monophyletic cluster with strong statistical support (BBP = 1), as expected since the species are in the same genus. *Epiphora bauhinia* grouped within the monophyletic tribe of Attacini, in agreement with its taxonomic placement. Both PCG123 and PCG12 trees were unable to recover the monophyletic tribal structure of Saturniini (**Figure 2.6**), as Saturniini species are split into three groups, of which two are monophyletic and *N. haraldi* appearing as a diverged branch.

Previous studies showed the non-monophyly of Saturniini and the incongruent position of *N. haraldi* (Saturniini); however, the splitting of Saturniini into two monophyletic clusters and the separation of *N. haraldi* from these two clusters was unexpected (He et al., 2017). In this study, I identified and corrected some errors in the mitogenomes of *N. haraldi* and *C. trifenestrata,* including artefactual single nucleotide indels which caused shifts in the reading frame of protein-coding genes. Even though I performed corrections, some errors may have escaped our curation, therefore the position *C. trifenestrata* and *N. haraldi* could be the result of substandard quality in these publicly available mitogenomes. All species from the genus *Saturnia,* together with *C. trifenestrata* and *E. pyretorum,* were recovered basal to Bunaeini (BPP = 0 .79) and are one of the two monophyletic clusters of the polyphyletic tribe Saturniini. The other monophyletic Saturniini cluster containing *Actias* and *Antheraea* species was recovered basal to Attacini with high nodal support (BPP = 0.93). It is also worth noting that some species were recovered as non-monophyletic: *Saturnia boisduvalii* (MF034742) was found as a sister to *Sa. jonasii* (MF034742) but not to the other *Sa. boisduvalii* sequence (NC_010613), and *Samia ricini* (NC_017869) was sister to *Samia canningi* (NC_024270) but not the other *S. ricini* (JF961379). The paraphyletic nature of these species could be a result of species misidentification or potential hybridization especially as closely related species like *S. ricini* and *S. canningi* have been hybridized to enhance their silk producing abilities (Brahma et al., 2011).

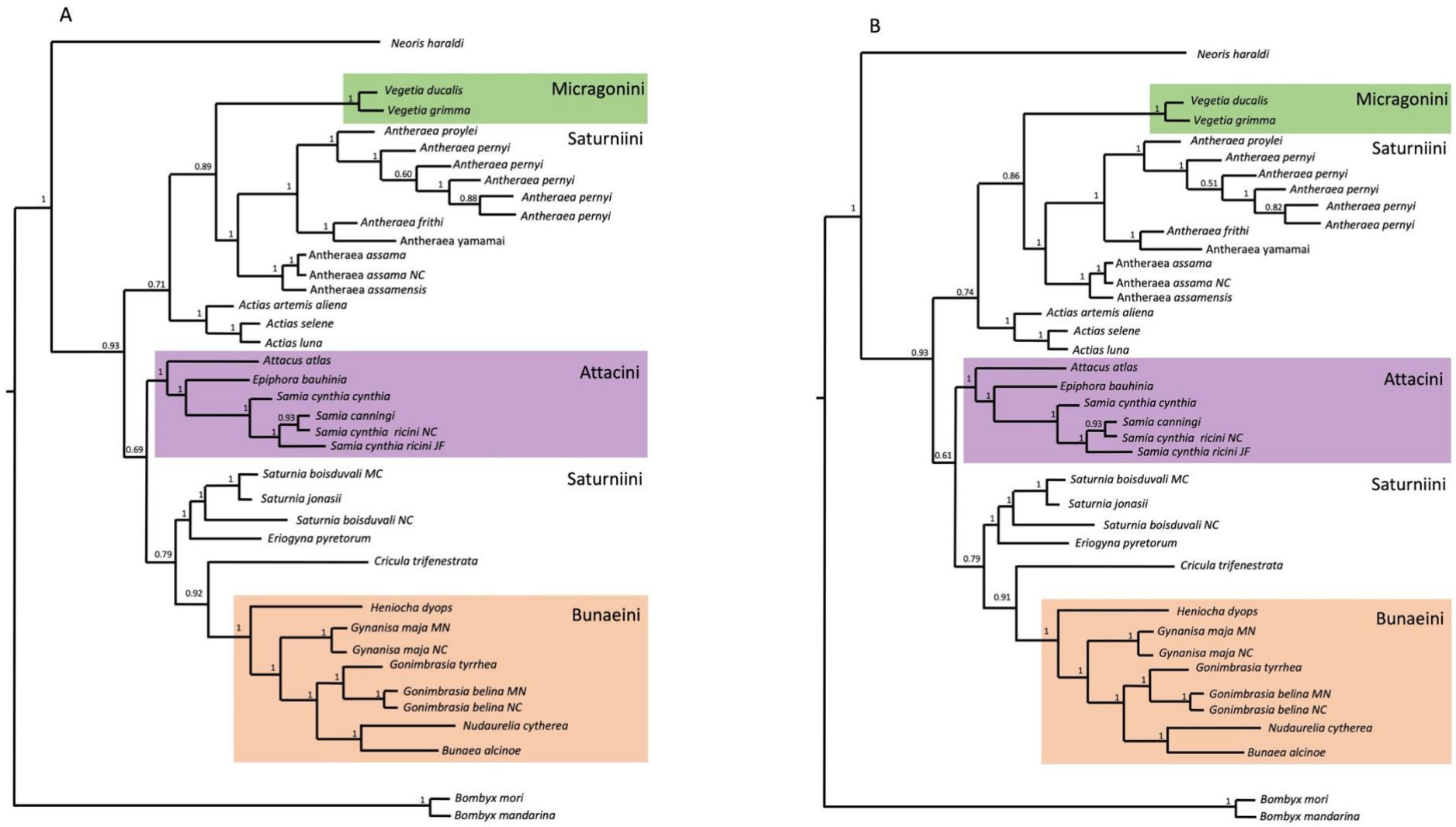

Figure 2.6 PCG123 and PCG12 phylogeny. Bayesian reconstruction of the mitochondrial phylogeny of Saturniidae based on 13 mitochondrial protein-coding genes (PCG). Nodal statistical supports are given Bayesian posterior probability. (A) PCG12 (1st and 2nd codon positions). (B) PCG123 (all codon positions).

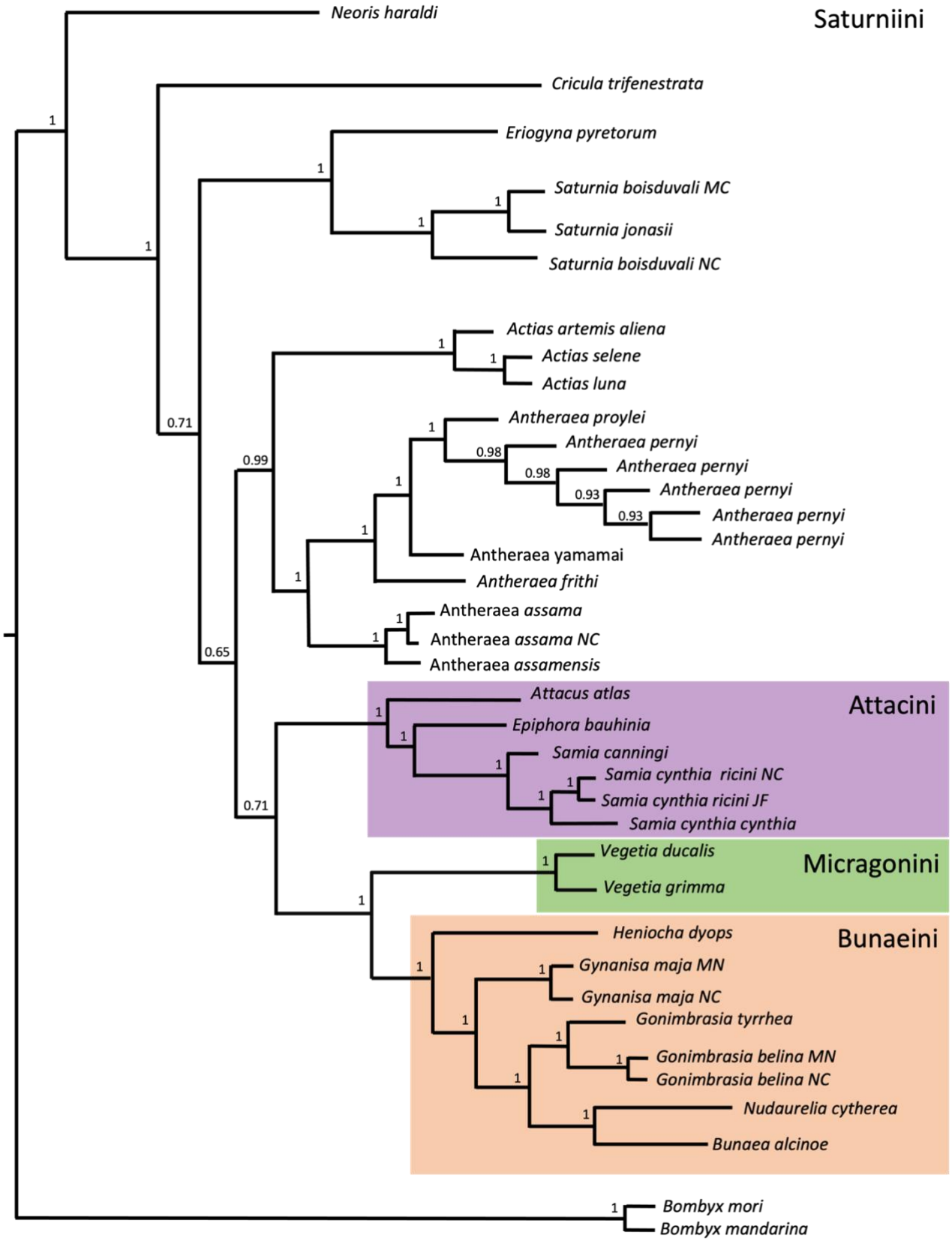

Figure 2.7 PCG123+rRNA. Bayesian reconstruction of the mitochondrial phylogeny of Saturniidae using all three codon positions of the total complement of 13 mitochondrial protein-coding genes and two rRNA genes (PCG123+rRNA). Nodal statistical support is given as Bayesian posterior probability.

The PCG123+rRNA tree recovered the same species clustering seen in the PCG123 and PCG12 trees (**Figure 2.7**; **Figure S 2**), which also was noted in previous studies, and Saturniini was also not monophyletic (Brahma et al., 2011; Kim et al., 2018; Langley et al., 2020). The PCG123+rRNA tree does not concur with the basal division of the subfamily Saturniidae dividing the Holarctic and Oriental tribes Saturniini and Attacini from the Afrotropical tribes Bunaeini, Urotini and Micragonini (Cameron, 2014; Regier et al., 2008). The inclusion of the two rRNA genes in addition to the 13 PCGs did not improve the recovery of taxonomic relationships in the family Saturniidae, although rRNA genes generally have lower mutation rate than mitochondrial PCGs, thus making them unsuitable as genetic markers for intraspecific studies but ideal for phylogenetic reconstructions (Mandal et al., 2014). Although this dataset significantly contributes to African Saturniidae mitogenomics, it covers less than 24% of all Southern African species, and therefore sub-Saharan Saturniidae mitogenomic information remains in short supply (Dube et al., 2013; Illgner et al., 2016; Langley et al., 2020).

2.5 Conclusion

Despite the addition of seven African Saturniidae mitogenomes newly presented in this study, the mitochondrial taxonomic coverage of the family Saturniidae remains lacking. All phylogenies failed to recover the monophyly of Saturniini. However, recovered Attacini, Bunaeini and Micragonini as monophyletic groups. Future phylogenetic reconstruction of the family Saturniidae should therefore include data derived from protein-coding nuclear genes in addition to mitochondrial genes, as nuclear DNA may provide insights into the order of the deeper nodes and potential phylogenetic patterns mediated by the male counterpart. Mitochondrial DNA lacks recombination due to its uniparental mode of inheritance, mtDNA markers thus cannot uncover deep divergence as they are unable to detected hybridization and introgression events, therefore the addition of nuclear markers is recommended. To further clarify the phylogenetic relationships in this family, mitogenomes from other species also need to be added, especially from African species. Although the first representatives from the tribe Micragonini have been included in this study, more clarity of phylogenetic relationships in the family would be possible via the inclusion of representatives from the tribes Urotini and Eochroini. When constructing phylogenies, one needs to be aware that some publicly available mitogenomes may have substandard sequence quality. I therefore suggest that future Saturniidae phylogenies excluded *C. trifenestrata* and *N. haraldi* as I detected and corrected numerous issues in their mitogenomes, including reading frame shifts thereby impacting their phylogenetic positioning within the Saturniidae family. This study therefore has significantly contributed to the clarification of evolutionary lineages within Saturniidae and has expanded the baseline genetic data available for African species, on which future genetic diversity, phylogeographic and phylogenetic studies can be based on.

2.6 Acknowledgements

The author is grateful to William Versfeld, Andrew Morton and Gail Morland for assistance with specimen collection. Kayleigh Hiscock is acknowledged for assistance with sequencing and assembling the mitogenomes of *V. ducalis* and *V. grimmia*.

Chapter 3: Phylogeography, genetic diversity and population structure of *Gonimbrasia belina* (mopane worm) in South Africa, Namibia and Botswana

3.1 Abstract

Gonimbrasia belina (Lepidoptera: Saturniidae) occurs in woodlands of *Colophospermum mopane* (mopane tree) and *Brachystegia* sp. (miombo tree) across southern Africa. *Gonimbrasia belina* is regarded as the most important edible Saturniidae caterpillar in the region as it provides not only food but also seasonal income for rural communities. In this study, I used two polymorphic mitochondrial amplicons (Amplicon A; 600 bp; located between ATP6 and COIII, and Amplicon C; 888 bp; located between ND6 and CYTB) in addition to the standard COI barcoding region (700 bp) to comparatively assess the genetic diversity, population structure and phylogeography of *G. belina* in three Southern African countries (South Africa, Botswana and Namibia). A total of 22 haplotypes were identified, with low levels of nucleotide diversity (0.0005) and high levels of haplotype diversity (0.77). High levels of genetic divergence were also detected among *G. belina* from the three countries with pairwise Fst values ranging from 0.58 to 0.75 ($P < 0.05$). The analysis of molecular variance (AMOVA) showed that a larger portion of molecular genetic variation is found among countries (61.29%) rather than within countries (38.71%). A maximum likelihood tree showed that most haplotypes clustered into three groups corresponding to the three countries, evidencing significant phylogeographic structure in *G. belina*. A haplotype network revealed that only one haplotype was shared by South Africa and Botswana. The findings from this study provide valuable information for future studies on the population structure of *G. belina,* and offer baseline data from which biodiversity hotspots may be identified in the future to inform sustainable harvesting and conservations plans.

3.2 Introduction

Gonimbrasia belina (Westwood,1894) is an edible Saturniidae (Lepidoptera) species which occurs in woodlands of *Colophospermum mopane* (mopane tree) and *Brachystegia sp.* (miombo tree) across southern Africa from the west to the east coast (Ghazoul, 2006). It is estimated that of the 2,000 edible insects reported worldwide, 18% are lepidopteran caterpillars (Goldstein, 2017). In Africa, 400 species of Saturniidae in 50 genera and six tribes have been identified to date. It is estimated that 50 to 70 of the total of 400 African Saturniidae species are consumed in central, south and western Africa (Oberprieler et al., 2021).

Despite improvements of living circumstances on the African continent, it still has the highest global occurrence of food insecurity, undernutrition and malnutrition (Halloran et al., 2015). Additionally, the African continent has the lowest protein per capita in the world, this is attributed to low income in rural areas which in turn limits access to protein (Sasson, 2012). Entomophagy provides essential nutrients to people in communities which have limited access to conventional protein sources (Makhado et al., 2014). Saturnids are an excellent conventional protein source alternative as they contain approximately three times the amount of protein (56.8 g) compared to beef (20 g) per 100 grams, and can be stored for months (Mutungi et al., 2019). The most important edible Saturniidae caterpillar in South Africa, Namibia and Botswana is *G. belina* as it provides not only food but seasonal income for rural communities (Illgner et al., 2016). In fact, vibrant international trade of *G. belina* has been established between Southern African countries such as Zambia, Zimbabwe, Botswana, South Africa and Namibia (Thomas, 2013). Edible caterpillars of Saturniidae species are generally known as “mopane worms” (among other local names). Mopane worm is the common name given to *G. belina* larvae and represents an important part of the diet and livelihoods of many communities due to its high market values and nutritional content (Ramos, 2005; Sasson, 2012).

Previous studies on mopane worms (*G. belina*) mainly focused on their potential for domestication, socio-economic, ecological and nutritional utilization (Hope et al., 2009; Thomas, 2013; Greenfield et al., 2014; Baiyegunhi et al., 2016). Most of these studies reported that the increased demand for mopane worms has led to overharvesting and serious decline of their wild population numbers. Some studies report such a severe decline that natural recovery seems unlikely (Roberts, 1998; Illgner et al.,2016). Genetic data for African Saturnids is mostly limited to DNA barcodes available on BOLD Systems and GenBank, and complete mitogenomic data exists for only 24% of the total number of species (see Chapter 2) (Langley et al., 2020). Studies of lepidopteran intraspecific genetic diversity and population structure have focused mainly on agricultural pests (Men et al., 2017). Despite the significance of mopane worms, their genetic diversity and phylogeographic structure has yet to be unravelled. Until the present work, only two studies have attempted to provide insight into the genetic structure and diversity of mopane worms (Greyling et al., 2001; Langley et al., 2020). The first study was performed in 2001 and provided insight into the genetic

diversity and structure of mopane worms utilizing allozymes to analyse a small number of *G. belina* specimens (Greyling et al., 2001). More recently, Langley et al. (2020) focused on identifying polymorphic mitochondrial regions which could be used as genetic markers in future studies to gain insight into the genetic diversity, phylogeography and population structure of *G. belina* (Langley et al., 2020).

Currently, the most widely used genomic resource in insects is still the mitochondrial genome (Cameron, 2014). Cytochrome c oxidase subunit I (COI) region is regarded as the most popular genetic marker for the identification of species as it has low intraspecific variation, high interspecific variation and highly conserved regions needed for primer annealing (Hebert et al., 2003). DNA barcoding is a popular method used to classify lepidopteran species, and it has been successful in resolving taxonomic problems and uncovering cryptic diversity in an extensive array of taxa (Burns et al., 2008; Lavinia et al., 2017). As COI does not provide sufficient resolution for intrapopulation and intraspecific studies due to low levels of polymorphism (Mandal et al., 2014), selection of more polymorphic mitochondrial markers is crucial. Optimal mitochondrial markers used for intraspecific studies require the following characteristics: highly polymorphic sites; have conserved regions for primer annealing; and sequences should be obtained through PCR amplicons of less than 1,200 bp. Ideally, mitochondrial markers should be located within PCGs, allowing for the identification of nuclear mitochondrial DNA (NUMTs), which may lead to incorrect inferences of genetic diversity (Thalman et al., 2004). An additional reason for the search for polymorphic mitochondrial markers to be restricted to PCGs is that it excludes rRNAs, which exhibit low mutation rate in insects thus are generally poor genetic markers for intraspecific studies (Mandal et al., 2014). Genetic diversity and structure of Lepidoptera are linked to host plant distribution, ability to migrate, geographic distance, climate fluctuations and geographical barriers (de Jong et al., 2011). Mopane worms are processed after harvesting either by drying or boiling, causing significant DNA degradation (Veldtman, 2019). Therefore, relatively short informative regions ranging from 600 bp to 888 bp are preferable, as they allow for the amplification of samples with low-quantity DNA (Langley et al., 2020). The utilization of polymorphic mitochondrial markers will help uncover genetic diversity and population structure in *G. belina* (Mutungi et al., 2019), which will aid in the identification of mopane harvesting sites, as the mopane worm trade is presently informal and untraceable (Makhado et al., 2014).

In this study, I used two polymorphic mitochondrial amplicons (Amplicon A; 600 bp; located between ATP6 and COIII, and Amplicon C; 888 bp; located between ND6 and CYTB) in addition to COI (700 bp) to investigate the genetic diversity, population structure and phylogeography of *G. belina* collected in South Africa, Botswana and Namibia.

3.3 Materials and methods

3.3.1 Sample collection and DNA extraction

A total of 115 caterpillars and adult specimens of *G. belina* (**Figure 3.1**) were collected, 51 from Botswana, 56 from South Africa and eight from Namibia between 15 March 2018 and 2021(**Figure 3.2**; **Table S3**). Specimens were euthanized by freezing within few hours of collection, whenever field conditions allowed, or refrigerated until further processing. Adult specimens were stored in sealed petri dishes at -20℃, and caterpillars were stored in 100% ethanol at room temperature until DNA extraction. DNA from the legs of caterpillars and adult specimens was extracted using a standard phenol-chloroform protocol (Barnett & Larson, 2012).

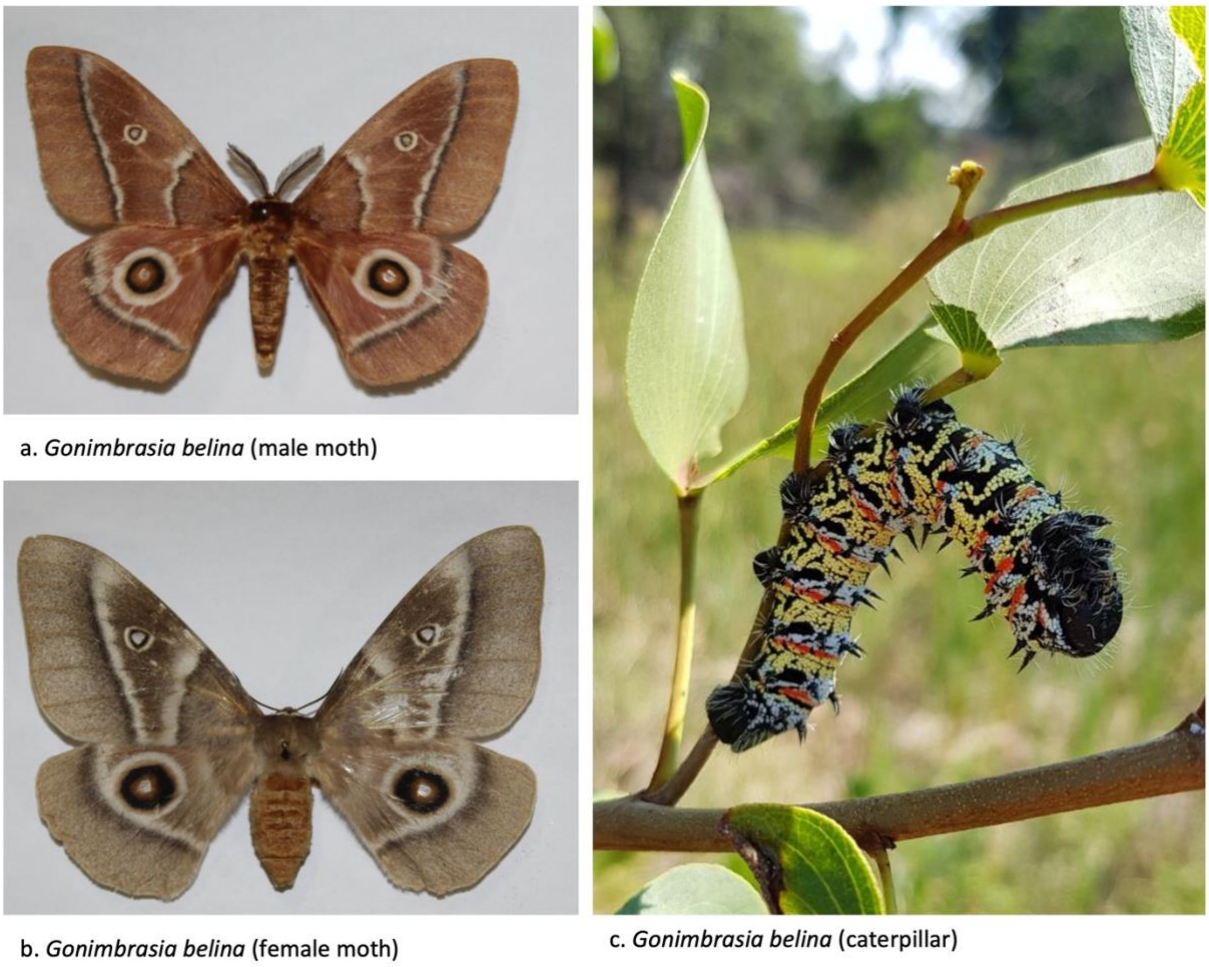

Figure 3.1 *Gonimbrasia belina* specimens. Adult and larval specimens of *Gonimbrasia belina* (Westwood, 1894). (A) *G. belina* (male moth), (B) *G. belina* (female moth) and (C) *G. belina* caterpillar (fifth instar larvae) feeding on mopane tree leaves.

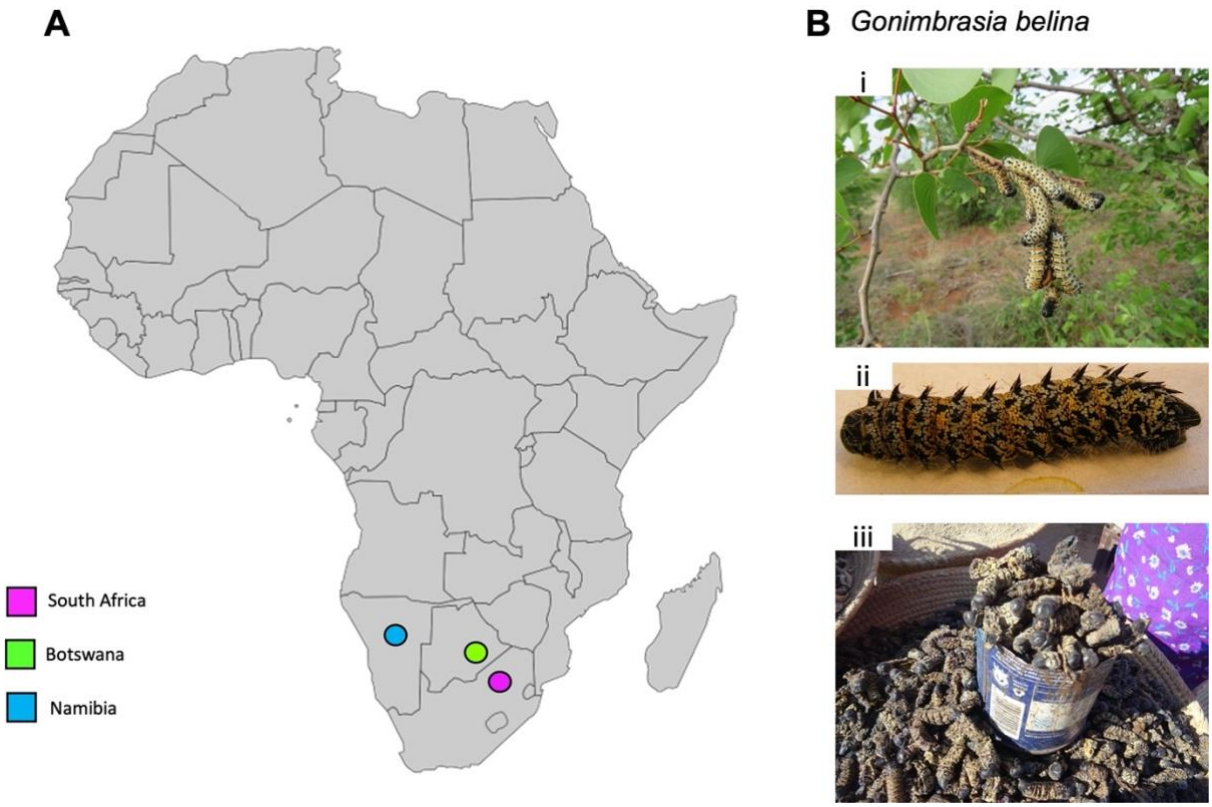

Figure 3.2 Approximate of sampling sites of *Gonimbrasia belina*. (Lepidoptera: Saturniidae). **(A)** Approximate location of *Gonimbrasia belina* collection sites in Botswana, Namibia, and South Africa. (B) (i) Caterpillars of *G. belina* feeding on mopane tree leaves (ii); Specimen preserved in ethanol (iii); Dried *G. belina* for sale at an informal market in Northern Namibia. Photo credits: John Mendelsohn, Ongava Research Centre.

3.3.2 DNA barcoding for species identification

Morphological identification of the specimens was performed by Rolf Oberprieler following the available literature and taxonomic keys (Pinhey, 1972; Cooper, 2002). To ensure that *G. belina* was accurately identified, I first sequenced the standard COI barcoding region (700 bp). The polymerase chain reaction (PCR) primers LepF and LepR were used as they are modifications of the Universal Folmer primers (LCO1490 and HCO2198), therefore have been specifically designed to amplify the barcoding region of Lepidoptera (Foottit et al., 2008). All PCR amplifications were performed in a total volume of 5 µL, containing 2.5 µL of Qiagen Multiplex PCR Kit (QIAGEN), 0.5 µL of each primer (0.5 µM), 0.5 µL of Milli-Q water and 1 µL of template DNA (~150 ng/ µL). Thermocycling conditions consisted of initial denaturation at 95°C for 15 min; 35 cycles of 94°C for 30 s, annealing at 54°C for 90 s and a final extension at 72°C for 10 min. PCR products which presented clean bands in a 1.5% agarose gel are sequenced unidirectionally using the BigDye

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), at the Central Analytical Facilities of Stellenbosch University. Sequences are manually edited and trimmed on Geneious Prime v2020.2.2 (https://www.geneious.com/) and aligned using the MAFFT algorithm plug-in (Katoh & Standley, 2013) on Geneious Prime. The barcodes produced were compared to all publicly available COI sequences for *G. belina* on BOLD and GenBank. To assess genetic clustering, sequence alignments were used to construct neighbour-joining (NJ) trees under the Kimura 2-parameter (K2P) model (Kimura, 1980) in MEGA X (Kumar et al., 2018). Intra- and interspecific distances were calculated in MEGA X as percentage of pairwise distances (p-distance, %) under the K2P model. Statistical support for the NJ trees and p-distances (standard error) were calculated using 1,000 bootstrap replicates.

3.3.3 Selection of mitochondrial genetic markers, PCR amplification and sequencing

Mitochondrial genetic marker selection was based on Langley et al. (2020) which found that the highest haplotype resolution for *G. belina* was achieved by concatenating two other polymorphic regions together with 700 bp standard barcoding COI region (Langley et al., 2020). Amplicon A is a 600 bp polymorphic region located within the mitochondrial ATP6 and COIII genes. The second polymorphic region is amplicon C, which is 888 bp and is located within ND6 and CYTB protein-coding genes.

PCR amplifications were performed using various primers (**Table 3.1**) in a total volume of 5 µL, containing 2x (2.5 µL) of Qiagen Multiplex PCR Kit (QIAGEN), 0.5 µL of each primer (0.5 µM), 0.5 µL of MilliQ water and 1 µL of template DNA (~100 ng/µL). Thermocycling conditions consisted of initial denaturation at 95°C for 15 min; 35 cycles of 94°C for 30 s, varying annealing temperatures for 90 s and a final extension at 72°C for 10 min (**Table 3.1**). PCR products which presented clear bands in a 1.5% agarose gel were sequenced bidirectionally for amplicon A and unidirectionally for amplicon C and the barcoding region using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), at the Central Analytical Facilities of Stellenbosch University. Sequences were manually edited and trimmed on Geneious Prime v2020.2.2 (https://www.geneious.com/) and aligned using MAFFT algorithm plug-in (Katoh & Standley, 2013) on Geneious Prime for each of the three amplicons. A 1,982 bp aligned sequence dataset was created by concatenating the barcoding region, Amplicon A and Amplicon C for all samples in Geneious Prime.

Table 3.1 Primer pairs and annealing temperatures. Primers and annealing temperatures (Ta) used for amplification of the standard barcoding COI region and the two polymorphic regions (Amplicon A (ATP6/COIII) and Amplicon B (ND6/CYTB)). *Primer used for Sanger sequencing.

Amplicon	Primer names	Primer sequence (5' – 3')	Ta (°C)	Gene region
DNA barcode	Lep F	ATTCAACCAATCATAAAGATATTGG	50	COI
	Lep R*	TAAACTTCTGGATGTCCAAAAAATCA		
A	GB-A3-F*	GATTATTTCCTTATATTTTCACTAG	56	ATP6
	GB-A3-R*	AACCTTTAATAACATAAATTGTATG		COIII
C	GB-C1-F	GTAGCAAGAATTGCCTCTAATG	52	ND6
	GB-C1-R*	AGCCTGTTTGATGAAGGAAAAG		CYTB

3.3.4 Mitochondrial diversity, population structure and neutrality test

Genetic diversity indices of each geographic population [number of haplotypes (*H*), haplotype diversity (*h*) and nucleotide diversity (π) differences] were calculated using Arlequin software v3.5 (Excoffier & Lischer, 2010). To test for population structure, *Gonimbrasia belina* specimens were grouped according to their country and an analysis of molecular variance (AMOVA) at 10,000 permutations was performed using Arlequin software v3.5 (Excoffier & Lischer, 2010). Tajima's D and Fu F's test was used to evaluate the demographic history of *G. belina* were also performed in Arlequin v3.5. P-values were generated using 1,000 simulations under the neutrality model (Fu, 1997).

3.3.5 Matrilineal phylogeny

The genetic clustering of 1,982 bp concatenated alignments (COI+ A+ C) of 115 *G. belina* was assessed using a maximum likelihood (ML) tree constructed in IQ-Tree (Minh et al., 2020). The best partitioning scheme (HKY+F+I; according to the Bayesian Information Criterion) was determined by automatic model selection (Schrempf et al., 2019). Nodal support was assessed using 1,000 bootstrap replications (Schrempf et al., 2019). The final ML tree was drawn using Figtree v1.4.4 (http://bio.tools/Figtree). To further illustrate evolutionary relationships among haplotypes, a median-joining network was constructed using Network v4.6.3, under the default settings (Bandelt et al., 1999).

3.4 Results

3.4.1 Sequence information

A total of 115 specimens were analysed, of which 51 from Botswana, 56 from South Africa and eight from Namibia. It is important to note that the number of *G. belina* specimens collected from each county is not equal.The final alignment included 1,982 bp from the three mitochondrial markers: DNA barcodes (700 bp), Amplicon A (600 bp) and Amplicon C (888 bp). The nucleotide composition of the concatenated sequences are very similar among the three countries the average A+T for Botswana, South Africa and Namibia 74.03%, 74.27% and 74.20%, respectively (**Table S4**). The A+T content (74%) for all three countries is higher than the average G+C content (26%) (**Figure 3.3**) evidencing the AT-bias that is commonly noted in lepidopteran species (Kim et al., 2018).

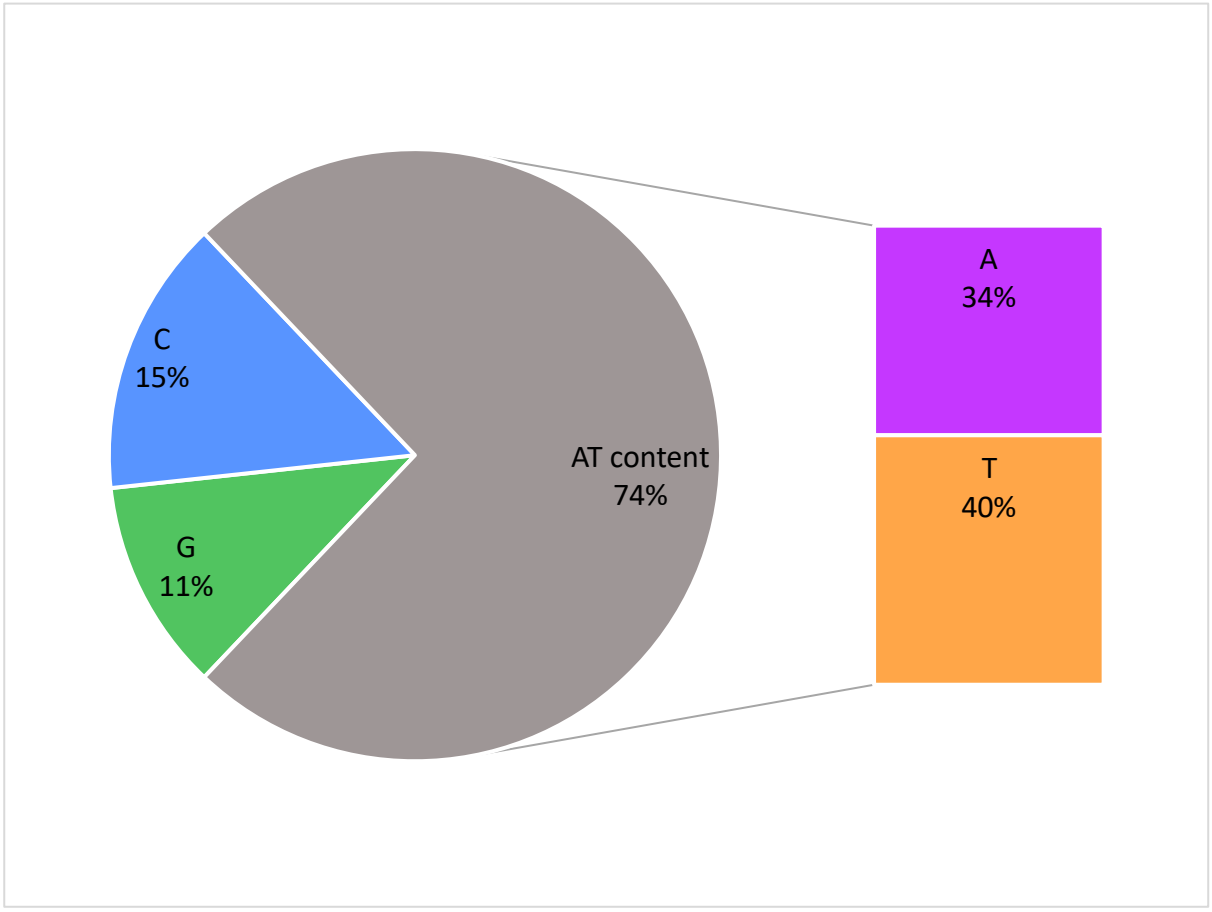

Figure 3.3 Nucleotide composition. Average nucleotide composition of *Gonimbrasia belina* from three geographic regions based on concatenated DNA barcodes (COI), Amplicon A (ATP6/COIII) and Amplicon C (ND6/CYTB) (total of 1,982 bp).

3.4.2 Geographic distribution of haplotypes

A total of 22 haplotypes were obtained from the complete dataset, of which 21 haplotypes are unique to their countries, and only haplotype (Hap1) is shared (between South Africa and Botswana) (**Figure 3.4**). Hap1 has the highest frequency and is present 54.7% of individuals in the dataset, and additionally has the highest frequency in Botswana (86.3% of individuals collected in the country). South Africa has the most haplotypes (n= 12), followed by Botswana (n= 8), and Namibia with only three haplotypes. Hap1 is the most frequent haplotype in South Africa (33.9%), followed by Hap14 (32.1%) and Hap11 (10.7%). In Namibia, two main haplotypes are present, with Hap22 observed in 62.5% of individuals and Hap20 in 25%.

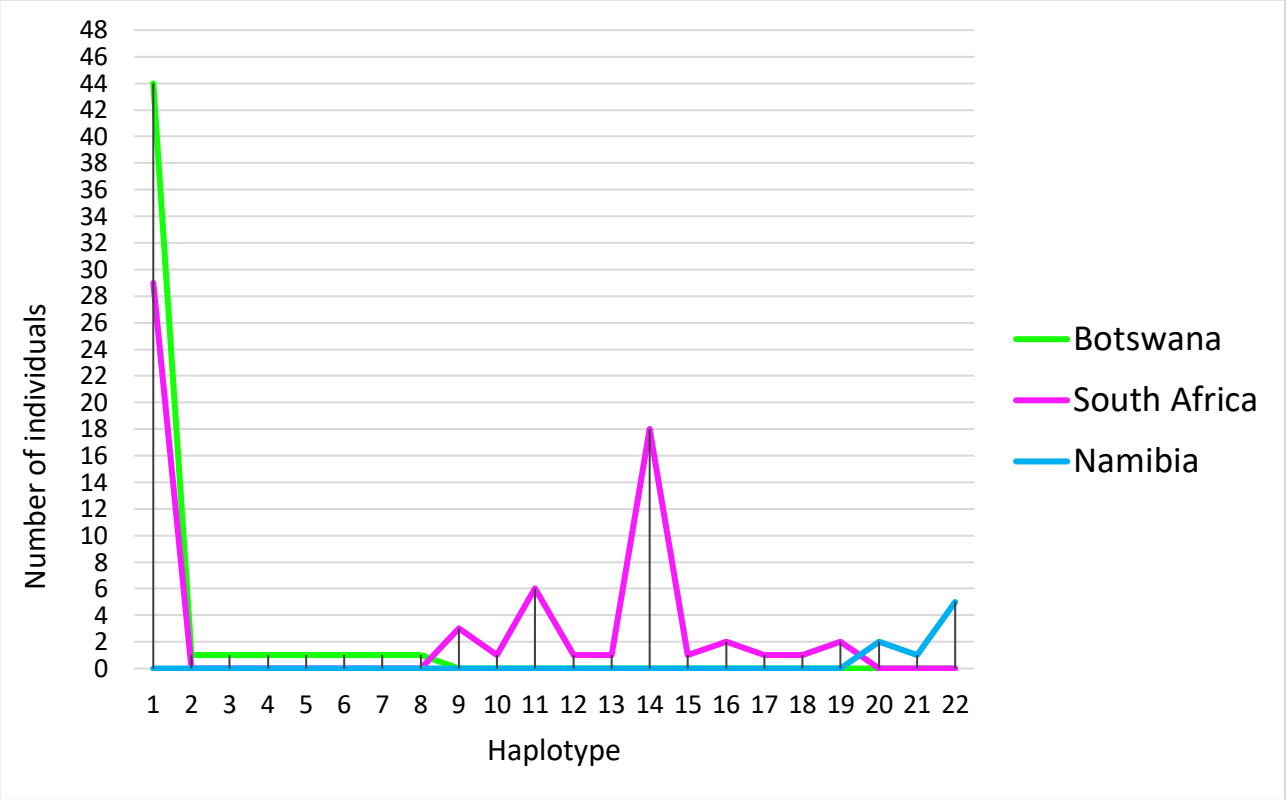

Figure 3.4 Haplotype frequency. Frequency of each of the 22 haplotypes of *Gonimbrasia belina* from South Africa, Botswana and Namibia based on concatenated DNA barcodes (COI), Amplicon A (ATP6-COIII) and Amplicon C (ND6-CYTB) (total = 1,982 bp).

3.4.3 Mitochondrial genetic diversity and structure

Overall, the haplotype diversity of *G. belina* ranges from 0.25 to 0.77, while the nucleotide diversity ranges from 0.0005 to 0.0195 (**Table 3.2**). The highest haplotype diversity is seen in *G. belina* from South Africa (0.77) and Namibia (0.67), while *G. belina* from Botswana has low haplotype diversity (0.25). All *G. belina* from the three geographic regions had low nucleotide diversity, the lowest observed in Namibia (0.0005) and Botswana (0.0027). *Gonimbrasia belina* from South Africa has

higher nucleotide diversity (0.0195) compared to Namibia (0.0005) and Botswana (0.0027). Nucleotide diversity and haplotype diversity of *G. belina* are both high in South Africa and low in Botswana, while in Namibia haplotype diversity is high and nucleotide diversity is low.

Table 3.2 Diversity indices. Diversity indices values for *Gonimbrasia belina* from South Africa, Namibia and Botswana based on concatenated mitochondrial genes COI, Amplicon A (ATP6-COIII) and Amplicon C (ND6-CYTB)). H - number of haplotypes per population, N - number of individuals, *h* - haplotype diversity, π - nucleotide diversity.

Country	**H**	**N**	***h***	***π***
Botswana	8	51	0.2580 ± 0.0814	0.0027 ± 0.0015
South Africa	12	56	0.7766 ± 0.0367	0.0195 ± 0.0095
Namibia	3	8	0.6071 ± 0.1640	0.0005 ± 0.0004

The highest genetic divergence is between *G. belina* from Namibia and South Africa, with pairwise Fst value of 0.75 ($P < 0.05$) (**Table 3.3**), in accordance with the significant geographic distance separating the sampling sites in those countries. Botswana and South Africa showed the lowest genetic divergence (0.58).

Table 3.3 Fst values. Pairwise genetic differentiation (Fst) between different geographic regions of *Gonimbrasia belina* (Botswana, South Africa and Namibia). Fst values are shown below the diagonal (level of significance at $P < 0.05$).

Country	**Botswana**	**South Africa**	**Namibia**
Botswana	0	-	-
South Africa	0.5819	0	-
Namibia	0.6383	0.7490	0

AMOVA results showed that a larger portion of molecular genetic variation is found among groups (61.29%) rather than within groups (38.71%) (**Table 3.4**). The AMOVA results support the median joining network's results, that *G. belina* is geographically structured into three regions: South Africa, Botswana and Namibia.

Table 3.4 Analysis of molecular variance (AMOVA). AMOVA of *Gonimbrasia belina* from three Southern African countries (South Africa, Namibia and Botswana) based on concatenated mitochondrial DNA genes COI, Amplicon A and Amplicon C. d.f - degrees of freedom. Fst significance $P < 0.05$.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variance
Among groups	2	999.95	15.12	61.29
Within groups	112	1,069.33	9.55	38.71
Total	114	2,064.28	24.66	100.00
Fixation index Fst			0.61	

3.4.4 Neutrality test

Neutrality tests were conducted using Tajima's D and Fu's F statistics for *G. belina* specimens from each of the three geographic regions. Tajima's D results were only significantly negative for specimens from Botswana (-2.106) and non-significantly positive for specimens from both South Africa (1.045) and Namibia (0.931) (**Table 3.5**). The Fu's F results for all geographic populations are positive ranging from 0.461 (Namibia) to 0.923 (Botswana).

Table 3.5 Neutrality test. Neutrality test (Tajima's D and Fu's Fs) values for *Gonimbrasia belina* from three Southern African countries (South Africa, Namibia and Botswana) based on concatenated mitochondrial DNA genes (COI, amplicon A (ATP6/COIII) and amplicon C (ND6/CYTB)). *Significant Tajima's D value ($P < 0.05$).

Country	Tajima's D	Fu's Fs
Botswana	-2.1067*	0.9230
South Africa	1.0447	0.9135
Namibia	0.9315	0.4610

3.4.5 Matrilineal phylogeny

The median-joining haplotype network evidenced strong phylogeographic structure in *G. belina* (**Figure 3.5**). The network shows a single shared haplotype (Hap1) between South Africa and Botswana. Additionally, it shows that most of the haplotypes (Hap2 to Hap22) present are unique and exclusive to their country of origin. Thereby, *G. belina* specimens are split into three distinct groups according to their geographic origin.

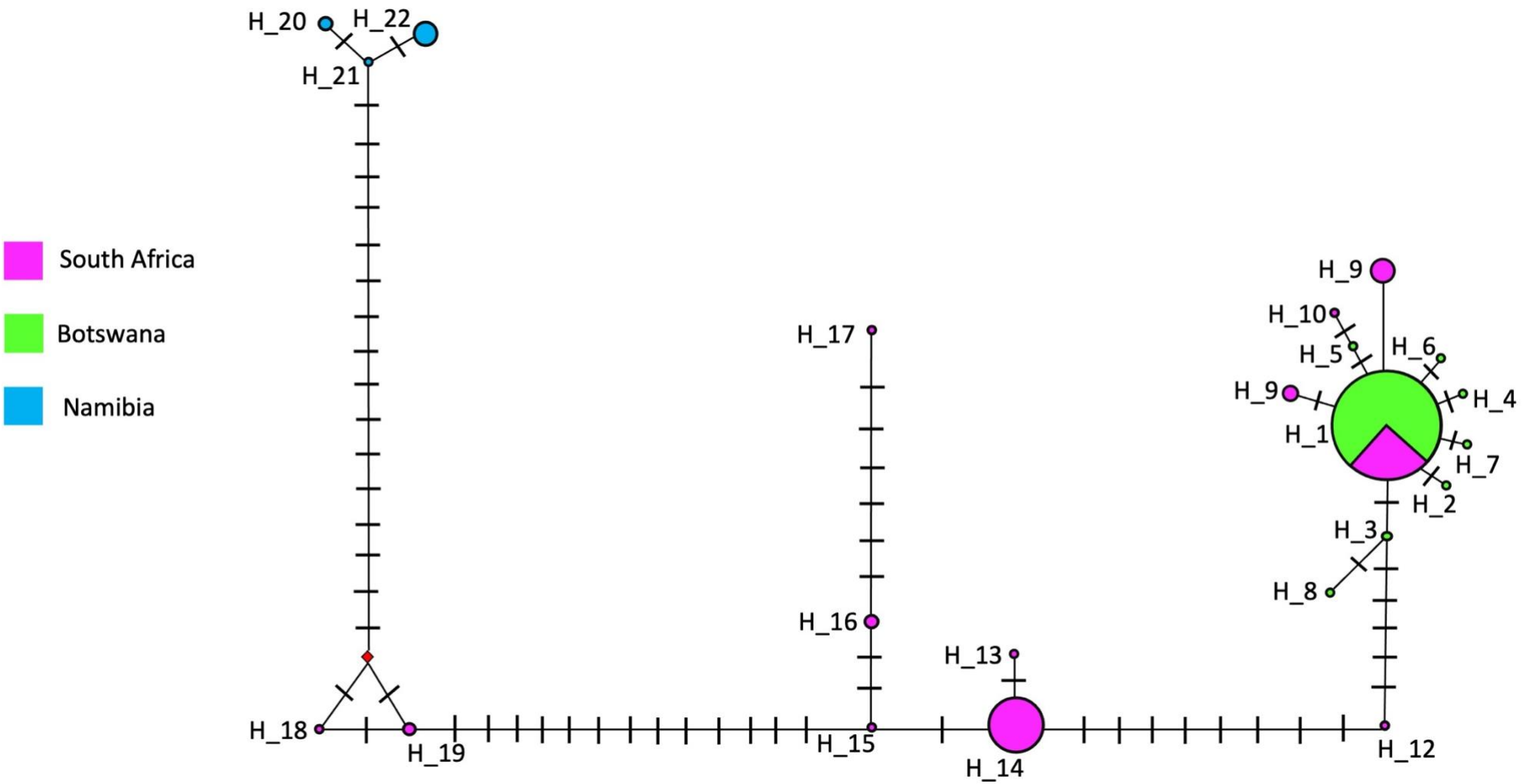

Figure 3.5 Median joining haplotype network. Median joining haplotype network of *Gonimbrasia belina* based on concatenated sequences of mitochondrial genes DNA barcodes (COI), Amplicon A (ATP6-COIII) and Amplicon C (ND6-CYTB). The circles are proportional to the frequency of the haplotype. The red diamond represents a hypothetical, unobserved haplotype. Short black lines represent the number of mutational steps separating each of the haplotypes.

The clustering of sequences on the ML tree is in accordance with the haplotype network, also showing strong phylogeographic structure (**Figure 3.6**). The 22 haplotypes cluster within their geographic locations and a clear division of haplotypes into their geographic location is evident. Haplotypes occurring in Namibia (Hap20-22) form a cluster clearly separated from haplotypes found in South Africa (Hap1, Hap9-19) and Botswana (Hap1-8). Additionally, the ML tree also shows the clustering of South African haplotypes with those from Botswana as well as the sharing of Hap1 between South Africa and Botswana.

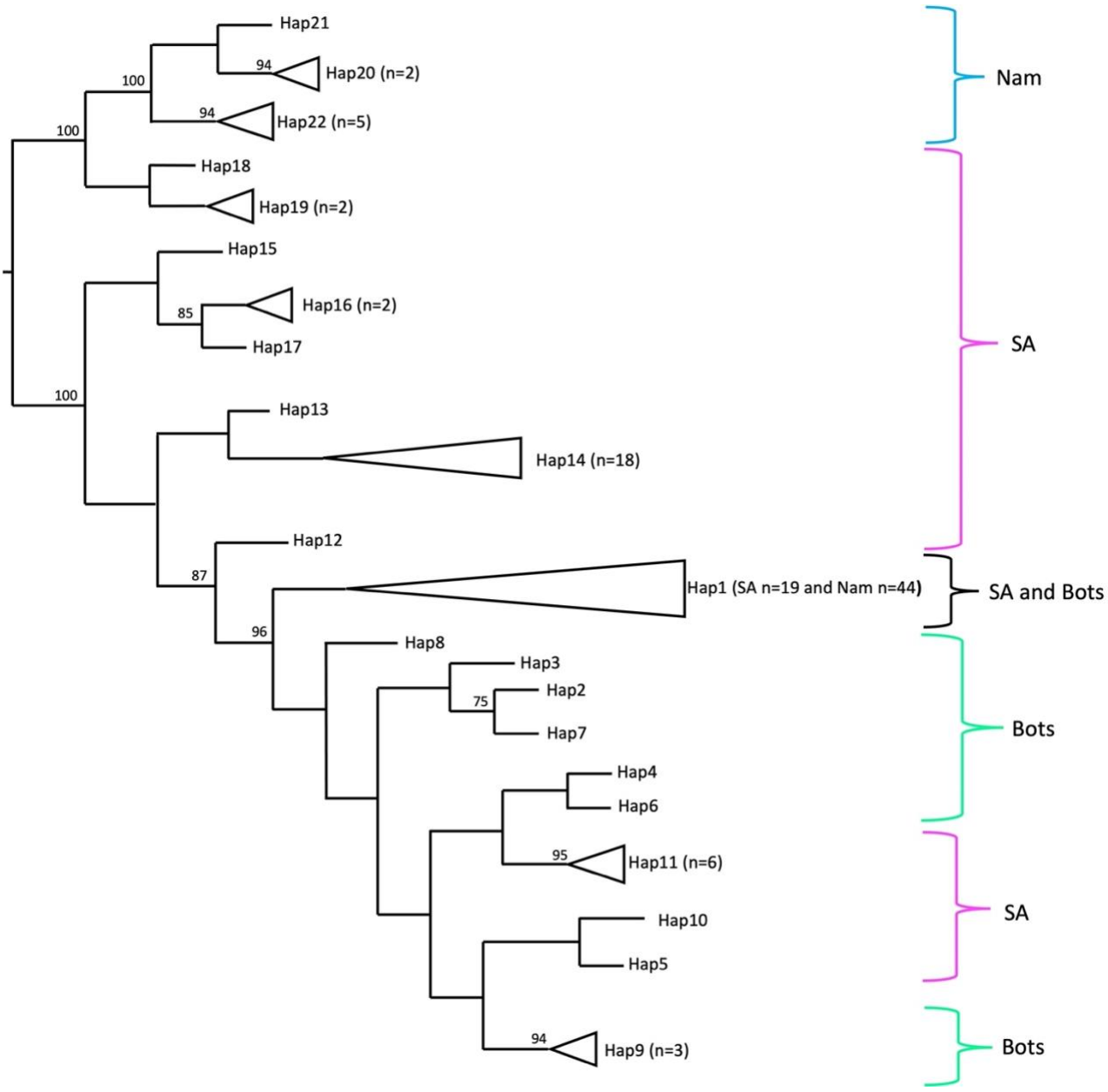

Figure 3.6 Maximum likelihood tree. Maximum likelihood tree of 22 mitochondrial haplotypes of *Gonimbrasia belina,* based on alignment of 1,982 bp, obtained from 115 individuals collected in Botswana (Bots, n= 51), South Africa (SA, n= 56) and Namibia (Nam, n= 8). Triangles represent collapsed groups of sequences belonging to the same haplotype. Nodal support was based on 1,000 bootstrap replicates (only values ≥ 75% were shown).

3.5 Discussion

In this study, I used three polymorphic mitochondrial gene regions (total of 1,982 bp) as markers to analyse the genetic structure and diversity of *G. belina* in three Southern African countries (South Africa, Botswana and Namibia) within their natural distribution range. The information gained by this study aimed at providing baseline data from which sustainable harvesting and effective conservation plans can be developed.

The basis for species adaptation, evolution and an important component of biodiversity is genetic diversity (Smith et al., 2022). Species with high genetic diversity are more adaptable to the environment (Diome et al., 2022; Smith et al., 2022). Genetic diversity is evaluated by haplotype and nucleotide diversity: the higher the value of these two indices, the higher the genetic diversity of the sequences in the dataset. Overall, *G. belina* from each of the three countries had high haplotype diversity. The most genetically diverse specimens were from South Africa and displayed the highest haplotype diversity and nucleotide diversity. Similarly high haplotype diversity have been reported in other lepidopteran species, like *Aglais urticae* and *Cameraria ohridella* (Men et al., 2017; Vandewoestijne et al., 2004). The least genetically diverse specimens were *G. belina* from Botswana with both low values of haplotype diversity and nucleotide diversity.

Low nucleotide diversity was observed in all three countries. However, *G. belina* in South Africa had higher nucleotide diversity than *G. belina* from Namibia or Botswana. Nucleotide diversity and haplotype diversity are both high in South Africa and low in Botswana, while haplotype diversity is high and nucleotide diversity is low in Namibia. Low nucleotide diversity and high haplotype diversity values indicate minimal differences between haplotypes and suggest expansion from a small effective population size (de Jong et al., 2011). Low haplotype and nucleotide diversity indicate low genetic diversity, therefore are a good indication of which population to focus conservation efforts on (Li et al., 2019).

Tajima's D and Fu's F statistics are significant when both are negative and are an indication that the population has undergone recent and rapid expansion (Men et al., 2017). When both values are non-significantly positive or one is positive and the other negative, the population has not experienced rapid expansion. Non-significant positive neutrality test values exhibited by *G. belina* in this study indicate the lack of population expansion (Men et al., 2017).The non-significant results of the neutrality tests are support by the median-joining haplotype network lacking a star shaped clustering pattern around a central haplotype which is an indication of historical population expansion (Ajao et al., 2021).

High numbers of unique haplotypes (21/22), a shortage of shared haplotypes and the lack of a central ancestral haplotype revealed by the median-joining haplotype network indicate that *G. belina* has strong phylogeographic structure (Goftishu et al., 2019). Additionally, the lack of population expansion observed in *G. belina* is supported by the presence of multiple unique

haplotypes (Lait & Hebert, 2018). The ML tree's haplotype clustering is similar to the haplotype clustering observed in the haplotype network. It is worth noting that results can be impacted by the small sample size of the Namibian specimens (n= 8). The number of samples needed to sufficiently detect alleles present within group is >30 (Wang et al., 2021). Additionally, 15 to 20 specimens can detect 90% of genetic diversity present (Wang et al., 2021). Therefore, it is important to note that sufficient number of specimens were collected from South Africa and Botswana (n= 56 and n= 51, respectively), while specimens from Namibia are under sampled and thereby could affect results.

Gonimbrasia belina is geographically structured into three regions: South Africa, Botswana and Namibia. *Gonimbrasia belina* are weak flyers, thus considered a sedentary lepidopteran species (Veldtman et al., 2007). Reduced gene flow is observed among sedentary lepidopteran species as a result of their weak flying ability (de Jong et al., 2011). Dispersal patterns of lepidopteran species significantly impact their genetic diversity and phylogeographic structure (Sekonya et al., 2020). Various sedentary lepidopterans species including *Chilo suppressalis*, *Carposina sasaki* and *Dendrolimus kikuchii* have exhibited similar trends (Men et al., 2017). In contrast, sufficient gene flow between populations prevents geographic divergence, and is identified by a lack of population structure over a large sampling area, such as that observed in Monarch butterflies (Zheng et al., 2013). Similarly, the haplotype structure of Western African honeybees (*Apis mellifera adansonii Latreille*) showed minimal to no geographic structure a result of high gene flow between populations (Ajao et al., 2021). The South African and Namibian sampling sites are separated from one another by approximately 1,600 km, thereby explaining the high genetic divergence observed between samples originating from these two countries. On the other hand, *G. belina* originating from Botswana and South Africa exhibited lower genetic divergence, likely due to the close proximity of the sampling sites in both countries (the closest sites were separated by approximately 3 km) that allows for gene flow to occur despite their weak flying ability, thus lower genetic divergence is observed. AMOVA results revealed majority of the variation in *G. belina* is between countries and not within, suggesting that the main cause of genetic divergence in *G. belina* observed is as a result of geographic isolation (Goftishu et al., 2019).

3.6 Conclusion

To the best of our knowledge, this is the first study to examine the genetic diversity and phylogeographic structure of *G. belina* using multiple polymorphic mitochondrial DNA markers and one of few studies involving Southern African Saturniidae. I found high genetic diversity in *G. belina* populations from Namibia and South Africa, and comparatively lower genetic diversity in Botswana. Genetic variation was mainly detected among countries. Significant phylogeographic structure was present in *G. belina*, with genetic divergence corresponding to the broad geographical area of origin of the specimen. These results provide valuable baseline information on the genetic diversity and population structure of *G. belina* from which future studies can be

based on. Furthermore, it provides baseline data for the future identification of trading and harvesting hotspots, which can aid in the development of conservation plans and management policies for sustainable harvesting of *G. belina* in southern Africa. I suggest that future studies on *G. belina* should include additional mitochondrial and nuclear markers, as nuclear DNA may provide insight into the migration and distribution of male moths. Additionally, by sampling more individuals and covering a greater distribution area of *G. belina* by including more countries within their distribution range, like Zimbabwe, Malawi, Zambia, Angola and Mozambique, will provide further detail on the population structure of this value alternative protein source. The addition of more countries will confirm if the phylogeographic structure observed in *G. belina* in this study extends throughout its Southern African distribution range. Based on the results from this study, I suggest that conservation efforts should be implemented in Botswana as both nucleotide diversity and haplotype diversity was low in that country (a sign of low genetic diversity). Low genetic diversity is a sign that numbers of *G. belina* in that region are declining.

3.7 Acknowledgements

The author is grateful to William Versfeld (Ongava Research Centre, Namibia) and Casper Nyamukondiwa (Botswana University of Science and Technology) for assistance with specimen collection.

Chapter 4: Conclusion

This study aimed to add to the phylogeny and expand the available genetic information of Southern African Saturniidae by sequencing and assembling mitogenomes of seven African Saturniidae species. Most of these African Saturniidae species are edible and important seasonal source of nutrition and income to various indigenous communities. Additionally, I focused on the most exploited edible African Saturniidae *G. belina*. I used multiple polymorphic mitochondrial markers to analysis and assess the genetic diversity and phylogeographic structure of *G. belina* in South Africa, Namibia and Botswana, three countries within their natural distribution range.

This study was comprised of three main chapters. Chapter one was the literature review, which highlighted the economic and culturally importance of Saturniidae globally and on the African continent. Additionally, the chapter revealed that African Saturniidae research has been neglected and introduced the key concepts explored by the present study. The second chapter aimed to generated baseline genetic data on African Saturniidae, by assessing the phylogenetic position of seven species: *H. dyops, G. tyrrhea, B. alcinoe, N. cytherea, E. bauhinia, V. ducalis* and *V. grimmia* in three tribes (Bunaeini, Micragonini and Attacini). This was achieved via mitochondrial genome sequencing and assembly. Chapter three focused on exploring the genetic diversity and phylogeography of *G. belina* in three Southern African countries using multiple mitochondrial markers.

4.1 Study outcomes

In Chapter two, the seven African Saturniidae mitogenomes sequenced and assembled by this study (*H. dyops, G. tyrrhea, B. alcinoe, N. cytherea, E. bauhinia, V. ducalis* and *V. grimmia*) significantly increased the mitochondrial taxonomic coverage of the family Saturniidae. The first representatives from the tribe Micragonini have been included in this study. Additionally in this chapter all our phylogenetic reconstruction of the Saturniidae family recovered the tribes Attacini, Bunaeini and Micragonini as monophyletic groups. All phylogenies failed to recover the monophyly of Saturniini. Thus, proving that the recovery of taxonomic relationships in the family Saturniidae did not improve when including the two rRNA genes in addition to the 13 PCGs.

Lastly chapter three, is the first study to examine the genetic diversity and phylogeographic structure of *G. belina* using multiple polymorphic mitochondrial DNA markers and one of few studies involving Southern African Saturniidae. Chapter three uncovered that *G. belina* presented strong phylogeographic structuring corresponding to the broad geographical area of origin of the specimens. The results in chapter three, not only provides valuable baseline information on the genetic diversity and population structure of *G. belina* from which future studies can be based on. It also provides baseline data for the identification of trading and harvesting hotspots, which is essential for the development of conservation plans and management policies for the sustainable harvesting of *G. belina* in Africa.

4.2 Limitations and prospects

I recommended that species identification should be based on both traditional morphological methods and DNA based methods. When constructing phylogenies, one needs to be aware that some publicly available mitogenomes may have substandard sequence quality. Mitogenomes with substandard sequence quality have reading frame shifts which in turn has an impact on the phylogenetic reconstruction. Furthermore, future studies can further clarify the phylogenetic relationships in the Saturniidae family by including representatives from the tribes Urotini and Eochroini.

The analysis of the genetic diversity and phylogeographic structure of *G. belina* in southern Africa provides valuable baseline information on the genetic diversity and population structure of *G. belina* from which future studies can be based on. Furthermore, it provides baseline data for the identification of trading and harvesting hotspots, which aids in development of conservation plans and management policies for sustainable harvesting of *G. belina* in Africa. The sampling of more individuals over a larger distribution area of *G. belina*, will provide further detail on the population structure of this value alternative protein source. Furthermore, the addition of more countries will confirm if the phylogeographic structure observed in *G. belina* extends throughout its Southern African distribution range.

Finally, I suggest that prospective studies should include nuclear markers in addition to mtDNA markers. Mitochondrial DNA lacks recombination due its uniparental mode of inheritance, mtDNA marker thus cannot uncover deep divergence as they are unable to detected hybridization and introgression events, nuclear DNA may therefore provide insights into the order of the deeper nodes, as well as migration and distribution of male moths.

Supplementary Data

Table S1 Main features of the complete mitogenomes. The main features of the complete mitogenomes of *Bunaea alcinoe, Epiphora bauhinia, Gonimbrasia tyrrhea, Heniocha dyops, Nudaurelia cytherea, Vegetia ducalis and Vegetia grimmia* (Lepidoptera: Saturniidae). N - minority strand; J - majority strand. IGN – number of intergenic nucleotides (overlapping genes are indicated by negative values).

Bunaea alcinoe								
Gene	**Code**	**Coordinates**	**Strand**	**Size (bp)**	**Anticodon**	**Start**	**Stop**	**IGN**
tRNA^{Met}	M	1-68	J	68	ATG	-	-	0
tRNA^{Ile}	I	67-130	J	64	ATC	-	-	-2
tRNA^{Gln}	G	127-198	J	72	CAA	-	-	-4
ND2	-	247-1,260	J	1,014	-	ATT	TAA	48
tRNA^{Trp}	W	1,262-1,330	J	69	TGA	-	-	1
tRNA^{Cys}	C	1,321-1,388	N	68	TGC	-	-	-10
tRNA^{Tyr}	Y	1,388-1,453	N	66	TAC	-	-	-1
COI	-	1,462-2,992	J	1,531	-	CGA	T--	8
tRNA^{Leu2}	L2	2,992-3,060	N	69	TTA	-	-	-1
COII	-	3,060-3,741	J	682	-	ATA	T--	-1
tRNA^{Lys}	K	3,741-3,813	J	73	AAG	-	-	-1
tRNA^{Asp}	D	3,833-3,900	J	68	GAC	-	-	19
ATP8	-	3,901-4,065	J	165	-	ATA	TAA	0
ATP6	-	4,059-4,736	J	678	-	ATG	TAA	-7
COXIII	-	4,736-5,524	J	789	-	ATG	TAA	0
tRNA^{Gly}	G	5,526-5,595	J	70	GGA	-	-	1
ND3	-	5,595-5,948	J	354	-	ATT	TAA	-1
tRNA^{Ala}	A	5,5958-6,026	J	69	GCA	-	-	9
tRNA^{Arg}	R	6,024-6,089	J	66	CGA	-	-	-3
tRNA^{Asn}	N	6,089-6,150	J	62	AAC	-	-	-1
tRNA^{Ser1}	S1	6,148-6,215	J	68	AGC	-	-	-3
tRNA^{Glu}	E	6,624-6,288	J	65	GAA	-	-	8
tRNA^{Phe}	F	6,287-6,354	N	68	TTC	-	-	-2
ND5	-	6,354-8,097	N	1,744	-	ATA	T--	-1
tRNA^{His}	H	8,097-8,163	N	67	CAC	-	-	-1
ND4	-	8,164-9,504	N	1,341	-	ATG	TAA	0
ND4L	-	9,504-9,794	N	291	-	ATG	TAA	-1
tRNA^{Thr}	T	9,800-9,867	J	68	ACA	-	-	5
tRNA^{Pro}	P	9,865-9,931	N	67	CCA	-	-	-3
ND6	-	9,933-10,466	J	534	-	ATA	TAA	1
CYTB	-	10,473-11,618	J	1,146	-	ATG	TAA	6
tRNA^{Ser2}	S2	11,630-11,692	J	63	TCA	-	-	11
ND1	-	11,748-12,686	N	939	-	ATT	TAA	55
tRNA^{Leu1}	L1	12,686-12,753	J	68	CTA	-	-	-1
16S rRNA		12,754-14,102	N	1,349	-	-	-	0
tRNA^{Val}	V	14,112-14,183	N	72	GTA	-	-	9
12S rRNA	-	14,184-14,941	N	758	-	-	-	-2
AT Rich region	-	14,942-15,305	J	-	-	-	-	0

Nudaurelia cytherea								
Gene	**Code**	**Coordinates**	**Strand**	**Size (bp)**	**Anticodon**	**Start**	**Stop**	**IGN**
$tRNA^{Met}$	M	1-65	J	65	ATG	-	-	0
$tRNA^{Ile}$	I	66-129	J	64	ATC	-	-	0
$tRNA^{Gln}$	G	126-196	J	71	CAA	-	-	-4
ND2	-	256-1,269	J	1,014	-	ATT	TAA	59
$tRNA^{Trp}$	W	1,272-1,343	J	72	TGA	-	-	2
$tRNA^{Cys}$	C	1,334-1,403	N	70	TGC	-	-	-10
$tRNA^{Tyr}$	Y	1,403-1,468	N	66	TAC	-	-	-1
COI	-	1,484-3,014	J	1,531	-	CGA	T--	15
$tRNA^{Leu2}$	L2	3,014-3,082	N	69	TTA	-	-	-1
COXII	-	3,082-3,763	J	682	-	ATA	T--	-1
$tRNA^{Lys}$	K	3,763-3,835	J	73	AAG	-	-	-1
$tRNA^{Asp}$	D	3,853-3,919	J	67	GAC	-	-	17
ATP8	-	3,920-4,084	J	165	-	ATT	TAA	0
ATP6	-	4,078-4,755	J	678	-	ATG	TAA	-7
COIII	-	4,755-5,543	J	789	-	ATG	TAA	-1
$tRNA^{Gly}$	G	5,545-5,614	J	70	GGA	-	-	1
ND3	-	5,614-5,967	J	354	-	ATT	TAA	-1
$tRNA^{Ala}$	A	5,972-6,043	J	72	GCA	-	-	4
$tRNA^{Arg}$	R	6,041-6,105	J	65	CGA	-	-	-3
$tRNA^{Asn}$	N	6,105-6,167	J	63	AAC	-	-	-1
$tRNA^{Ser1}$	S1	6,166-6,235	J	70	AGC	-	-	-2
$tRNA^{Glu}$	E	6,241-6,320	J	80	GAA	-	-	5
$tRNA^{Phe}$	F	6,308-6,377	N	70	TTC	-	-	-13
ND5	-	6,377-8,117	N	1,741	-	ATA	TA-	-1
$tRNA^{His}$	H	8,117-8,186	N	70	CAC	-	-	-1
ND4	-	8,188-9,528	N	1,341	-	ATG	TAA	1
ND4L	-	9,528-9,818	N	291	-	ATG	TAA	-1
$tRNA^{Thr}$	T	9,821-9,888	J	68	ACA	-	-	2
$tRNA^{Pro}$	P	9,886-9,954	N	69	CCA	-	-	-3
ND6	-	9,956-10,486	J	531	-	ATA	TAA	1
CYTB	-	10,492-11,637	J	1,146	-	ATG	TAA	5
$tRNA^{Ser2}$	S2	11,686-11,752	J	67	TCA	-	-	48
ND1	-	11,771-12,709	N	939	-	ATT	TAA	18
$tRNA^{Leu1}$	L1	12,709-12,776	J	68	CTA	-	-	-1
16S rRNA		12,777-14,165	N	1,389	-	-	-	0
$tRNA^{Val}$	V	14,166-14,320	N	65	GTA	-	-	0
12S rRNA	-	14,999-14,231	N	769	-	-	-	0
AT Rich region	-	15,000-15,363	J	-	-	-	-	0

Gonimbrasia tyrrhea								
Gene	**Code**	**Coordinates**	**Strand**	**Size (bp)**	**Anticodon**	**Start**	**Stop**	**IGN**
$tRNA^{Met}$	M	1-70	J	70	ATG	-	-	0
$tRNA^{Ile}$	I	68-132	J	65	ATC	-	-	-3
$tRNA^{Gln}$	G	129-199	J	71	CAA	-	-	-4
ND2	-	252-1,265	J	1,014	-	ATT	TAA	52
$tRNA^{Trp}$	W	1,268-1,338	J	71	TGA	-	-	2
$tRNA^{Cys}$	C	1,329-1,396	N	68	TGC	-	-	-10
$tRNA^{Tyr}$	Y	1,396-1,461	N	66	TAC	-	-	-1
COI	-	1,469-2,999	J	1,531	-	CGA	T--	7
$tRNA^{Leu2}$	L2	2,999-3,065	N	67	TTA	-	-	-1
COII	-	3,067-3,748	J	682	-	ATA	T--	1
$tRNA^{Lys}$	K	3,748-3,820	J	73	AAG	-	-	-1
$tRNA^{Asp}$	D	3,843-3,912	J	70	GAC	-	-	22
ATP8	-	3,913-4,077	J	165	-	ATT	TAA	0
ATP6	-	4,071-4,748	J	678	-	ATG	TAA	-7
COIII	-	47,48-5,536	J	789	-	ATG	TAA	-1
$tRNA^{Gly}$	G	5,538-5,606	J	69	GGA	-	-	1
ND3	-	5,606-5,959	J	354	-	ATT	TAA	-1
$tRNA^{Ala}$	A	5,963-6,030	J	68	GCA	-	-	3
$tRNA^{Arg}$	R	6,029-6,097	J	69	CGA	-	-	-2
$tRNA^{Asn}$	N	6,097-6,159	J	63	AAC	-	-	-1
$tRNA^{Ser1}$	S1	6,157-6,226	J	70	AGC	-	-	-3
$tRNA^{Glu}$	E	6,230-6,295	J	66	GAA	-	-	3
$tRNA^{Phe}$	F	6,296-6,363	N	68	TTC	-	-	0
ND5	-	6,363-8,106	N	1,744	-	ATA	T--	-1
$tRNA^{His}$	H	8,106-8,175	N	70	CAC	-	-	-1
ND4	-	8,180-9,520	N	1,341	-	ATG	TAG	4
ND4L	-	9,523-9,813	N	291	-	ATG	TAA	2
$tRNA^{Thr}$	T	9,816-9,883	J	68	ACA	-	-	2
$tRNA^{Pro}$	P	9,881-9,949	N	69	CCA	-	-	-3
ND6	-	9,951-10,484	J	534	-	ATA	TAA	1
CYTB	-	10,490-11,641	J	1,152	-	ATG	TAA	5
$tRNA^{Ser2}$	S2	11,679-11,745	J	67	TCA	-	-	37
ND1	-	11,763-12,701	N	939	-	ATT	TAA	17
$tRNA^{Leu1}$	L1	12,701-12,768	J	68	CTA	-	-	-1
16S rRNA		12,769-14,126	N	1,358	-	-	-	0
$tRNA^{Val}$	V	14,127-14,193	N	67	GTA	-	-	0
12S rRNA	-	14,194-14,948	N	755	-	-	-	0
AT Rich region	-	14,949-15,299	J	-	-	-	-	0

Epiphora bauhinia								
Gene	**Code**	**Coordinates**	**Strand**	**Size (bp)**	**Anticodon**	**Start**	**Stop**	**IGN**
$tRNA^{Met}$	M	1-68	J	68	ATG	-	-	0
$tRNA^{Ile}$	I	76-139	J	64	ATC	-	-	7
$tRNA^{Gln}$	G	136-206	J	71	CAA	-	-	-4
ND2	-	253-1,264	J	1,012	-	ATT	T--	46
$tRNA^{Trp}$	W	1,264-1,334	J	71	TGA	-	-	-1
$tRNA^{Cys}$	C	1,326-1,387	N	62	TGC	-	-	-9
$tRNA^{Tyr}$	Y	1,384-1,451	N	68	TAC	-	-	-4
COI	-	1,461-2,991	J	1,531	-	CGA	T--	9
$tRNA^{Leu2}$	L2	2,991-3,058	N	68	TTA	-	-	-1
COII	-	3,058-3,739	J	682	-	ATG	T--	-2
$tRNA^{Lys}$	K	3,739-3,811	J	73	AAG	-	-	-1
$tRNA^{Asp}$	D	3,827-3,898	J	72	GAC	-	-	15
ATP8	-	3,898-4,062	J	165	-	ATC	TAA	-1
ATP6	-	4,056-4,733	J	678	-	ATG	TAA	-7
COIII	-	4,733-5,521	J	789	-	ATG	TAA	-1
$tRNA^{Gly}$	G	5,523-5,590	J	68	GGA	-	-	1
ND3	-	5,590-5,941	J	352	-	ATT	T--	-1
$tRNA^{Ala}$	A	5,941-6,011	J	71	GCA	-	-	-1
$tRNA^{Arg}$	R	6,009-6,074	J	66	CGA	-	-	-3
$tRNA^{Asn}$	N	6,074-6,138	J	68	AAC	-	-	-1
$tRNA^{Ser1}$	S1	6,139-6,208	J	70	AGC	-	-	0
$tRNA^{Glu}$	E	6,207-6,275	J	69	GAA	-	-	-2
$tRNA^{Phe}$	F	6,272-6,340	N	69	TTC	-	-	-4
ND5	-	6,340-8,083	N	1,744	-	ATT	T--	-1
$tRNA^{His}$	H	8,083-8,150	N	68	CAC	-	-	-1
ND4	-	8,150-9,490	N	1,341	-	ATG	TAA	-1
ND4L	-	9,490-9,780	N	291	-	ATG	TAA	-1
$tRNA^{Thr}$	T	9,875-9,850	J	66	ACA	-	-	4
$tRNA^{Pro}$	P	9,850-9,916	N	67	CCA	-	-	-1
ND6	-	9,918-10,448	J	531	-	ATA	TAA	1
CYTB	-	10,455-11,603	J	1,149	-	ATG	TAA	6
$tRNA^{Ser2}$	S2	11,605-11,670	J	66	TCA	-	-	-1
ND1	-	11,691-12,629	N	939	-	ATG	TAA	20
$tRNA^{Leu1}$	L1	12,631-12,698	J	68	CTA	-	-	1
16S rRNA		12,699-14,054	N	1,356	-	-	-	0
$tRNA^{Val}$	V	14,055-14,123	N	69	GTA	-	-	0
12S rRNA	-	14,124-14,902	N	779	-	-	-	0
AT Rich region	-	14,903-15,259	J	-	-	-	-	0

Heniocha dyops								
Gene	**Code**	**Coordinates**	**Strand**	**Size (bp)**	**Anticodon**	**Start**	**Stop**	**IGN**
$tRNA^{Met}$	M	1-65	J	65	ATG	-	-	0
$tRNA^{Ile}$	I	61-126	J	66	ATC	-	-	-5
$tRNA^{Gln}$	G	122-191	J	70	CAA	-	-	-5
ND2	-	242-1,255	J	1,014	-	ATC	TAA	50
$tRNA^{Trp}$	W	1,253-1,322	J	70	TGA	-	-	-3
$tRNA^{Cys}$	C	1,314-1,376	N	63	TGC	-	-	-9
$tRNA^{Tyr}$	Y	1,376-1,441	N	66	TAC	-	-	-1
COI	-	1,449-2,979	J	1,531	-	CGA	T--	7
$tRNA^{Leu2}$	L2	2,979-3,047	N	69	TTA	-	-	-1
COII	-	3,047-3,728	J	682	-	ATA	T--	-1
$tRNA^{Lys}$	K	3,728-3,800	J	73	AAG	-	-	-1
$tRNA^{Asp}$	D	3,828-3,898	J	71	GAC	-	-	27
ATP8	-	3,898-4,068	J	171	-	ATT	TAA	-1
ATP6	-	4,062-4,739	J	678	-	ATG	TAA	-7
COIII	-	4,739-5,527	J	789	-	ATG	TAA	-1
$tRNA^{Gly}$	G	5,532-5,597	J	66	GGA	-	-	4
ND3	-	5,597-5,950	J	354	-	ATT	TAA	-1
$tRNA^{Ala}$	A	5,955-6,024	J	70	GCA	-	-	4
$tRNA^{Arg}$	R	6,022-6,086	J	65	CGA	-	-	-3
$tRNA^{Asn}$	N	6,098-6,165	J	68	AAC	-	-	11
$tRNA^{Ser1}$	S1	6,166-6,233	J	68	AGC	-	-	0
$tRNA^{Glu}$	E	6,232-6,299	J	68	GAA	-	-	-2
$tRNA^{Phe}$	F	6,295-6,362	N	68	TTC	-	-	-5
ND5	-	6,364-8,106	N	1,743	-	ATT	TAA	1
$tRNA^{His}$	H	8,106-8,172	N	67	CAC	-	-	-1
ND4	-	8,173-9,513	N	1,341	-	ATG	TAA	0
ND4L	-	9,515-9,805	N	291	-	ATG	TAA	1
$tRNA^{Thr}$	T	9,811-9,879	J	69	ACA	-	-	5
$tRNA^{Pro}$	P	9,877-9,943	N	67	CCA	-	-	-3
ND6	-	9,945-10,478	J	534	-	ATA	TAA	1
CYTB	-	10,484-11,635	J	1,152	-	ATG	TAA	5
$tRNA^{Ser2}$	S2	11,676-11,741	J	66	TCA	-	-	40
ND1	-	11,760-12,698	N	939	-	ATG	TAA	18
$tRNA^{Leu1}$	L1	12,699-12,766	J	68	CTA	-	-	0
16S rRNA		12,767-14,124	N	1,358	-	-	-	0
$tRNA^{Val}$	V	14,125-14,192	N	68	GTA	-	-	0
12S rRNA	-	14,913-14,945	N	753	-	-	-	0
AT Rich region	-	14,946-15,306	J	-	-	-	-	0

Vegetia ducalis								
Gene	**Code**	**Coordinates**	**Strand**	**Size (bp)**	**Anticodon**	**Start**	**Stop**	**IGN**
tRNA^{Met}	M	1-69	J	69	ATG	-	-	0
tRNA^{Ile}	I	68-131	J	64	ATC	-	-	-2
tRNA^{Gln}	G	128-198	J	71	CAA	-	-	-4
ND2	-	246-1,259	J	1,014	-	ATT	TAA	47
tRNA^{Trp}	W	1,268-1,334	J	67	TGA	-	-	8
tRNA^{Cys}	C	1,326-1,392	N	67	TGC	-	-	-9
tRNA^{Tyr}	Y	1,393-1,456	N	64	TAC	-	-	0
COI	-	1,460-2,990	J	1,531	-	CGA	T--	3
tRNA^{Leu2}	L2	2,990-3,060	N	71	TTA	-	-	-1
COII	-	3,061-3,742	J	682	-	ATG	T--	0
tRNA^{Lys}	K	3,742-3,814	J	73	AAG	-	-	-1
tRNA^{Asp}	D	3,833-3,899	J	67	GAC	-	-	18
ATP8	-	3,900-4,064	J	165	-	ATT	TAA	0
ATP6	-	4,058-4,735	J	678	-	ATG	TAA	-7
COIII	-	4,735-5,523	J	789	-	ATG	TAA	-1
tRNA^{Gly}	G	5,525-5,592	J	68	GGA	-	-	1
ND3	-	5,589-5,945	J	357	-	ATA	TAA	-4
tRNA^{Ala}	A	5,946-6,013	J	68	GCA	-	-	0
tRNA^{Arg}	R	6,011-6,075	J	65	CGA	-	-	-3
tRNA^{Asn}	N	6,075-6,139	J	65	AAC	-	-	-1
tRNA^{Ser1}	S1	6,137-6,206	J	70	AGC	-	-	-3
tRNA^{Glu}	E	6,205-6,272	J	68	GAA	-	-	-2
tRNA^{Phe}	F	6,273-6,342	N	701	TTC	-	-	0
ND5	-	6,348-8,076	N	1,729	-	ATT	T--	5
tRNA^{His}	H	8,077-8,140	N	64	CAC	-	-	0
ND4	-	8,148-9,482	N	1,335	-	ATG	TAA	7
ND4L	-	9,843-9,773	N	291	-	TAA	TA-	0
tRNA^{Thr}	T	9,780-9,844	J	65	ACA	-	-	6
tRNA^{Pro}	P	9,844-9,909	N	66	CCA	-	-	-1
ND6	-	9,911-10,438	J	528	-	ATA	TAA	1
CYTB	-	10,446-11,591	J	1,146	-	ATG	TAA	7
tRNA^{Ser2}	S2	11,608-11,672	J	65	TCA	-	-	16
ND1	-	11,691-12,629	N	939	-	ATG	TAA	18
tRNA^{Leu1}	L1	12,631-12,700	J	70	CTA	-	-	1
16S rRNA		12,701-14,046	N	1,346	-	-	-	0
tRNA^{Val}	V	14,057-14,124	N	68	GTA	-	-	10
12S rRNA	-	14,125-14,880	N	756	-	-	-	0
AT Rich region	-	14,881-15,218	J	-	-	-	-	0

Vegetia grimmia								
Gene	**Code**	**Coordinates**	**Strand**	**Size (bp)**	**Anticodon**	**Start**	**Stop**	**IGN**
$tRNA^{Met}$	M	1-66	J	66	ATG	-	-	0
$tRNA^{Ile}$	I	69-132	J	69	ATC	-	-	2
$tRNA^{Gln}$	G	129-199	J	71	CAA	-	-	-4
ND2	-	247-1,260	J	1,014	-	ATT	TAA	47
$tRNA^{Trp}$	W	1,269-1,335	J	67	TGA	-	-	8
$tRNA^{Cys}$	C	1,327-1,392	N	66	TGC	-	-	-9
$tRNA^{Tyr}$	Y	1,401-1,464	N	64	TAC	-	-	8
COI	-	1,468-2,998	J	1,531	-	CGA	T--	3
$tRNA^{Leu2}$	L2	2,998-3,068	N	71	TTA	-	-	-1
COII	-	3,069-3,750	J	682	-	ATG	T--	0
$tRNA^{Lys}$	K	3,750-3,822	J	73	AAG	-	-	-1
$tRNA^{Asp}$	D	3,841-3,908	J	68	GAC	-	-	18
ATP8	-	3,909-4,070	J	162	-	ATT	TAA	0
ATP6	-	4,064-4,741	J	678	-	ATG	TAA	-7
COIII	-	4,741-5,529	J	789	-	ATG	TAA	-1
$tRNA^{Gly}$	G	5,531-5,599	J	69	GGA	-	-	1
ND3	-	5,596-5,952	J	357	-	ATA	TAA	-4
$tRNA^{Ala}$	A	5,953-6,019	J	67	GCA	-	-	0
$tRNA^{Arg}$	R	6,017-6,081	J	65	CGA	-	-	-3
$tRNA^{Asn}$	N	6,081-6,145	J	65	AAC	-	-	-1
$tRNA^{Ser1}$	S1	6,143-6,212	J	70	AGC	-	-	-3
$tRNA^{Glu}$	E	6,211-6,278	J	68	GAA	-	-	-2
$tRNA^{Phe}$	F	6,279-6,348	N	70	TTC	-	-	0
ND5	-	6,347-8,085	N	1,739	-	TAA	TA-	-2
$tRNA^{His}$	H	8,086-8,149	N	64	CAC	-	-	0
ND4	-	8,151-9,491	N	1,341	-	ATG	TAA	1
ND4L	-	9,492-9,782	N	291	-	ATG	TTA	0
$tRNA^{Thr}$	T	9,789-9,853	J	65	ACA	-	-	6
$tRNA^{Pro}$	P	9,853-9,918	N	66	CCA	-	-	-1
ND6	-	9,920-10,450	J	531	-	ATA	TAA	1
CYTB	-	10,450-11,616	J	1,167	-	ATT	TAA	-1
$tRNA^{Ser2}$	S2	11,620-11,684	J	65	TCA	-	-	3
ND1	-	11,703-12,641	N	939	-	ATG	TAA	18
$tRNA^{Leu1}$	L1	12,643-12,713	J	71	CTA	-	-	1
16S rRNA		12,710-14,059	N	1,350	-	-	-	-4
$tRNA^{Val}$	V	140,71-14,138	N	68	GTA	-	-	11
12S rRNA	-	14,136-14,894	N	759	-	-	-	-3
AT Rich region	-	14,895-15,253	J	-	-	-	-	0

Table S2 Complete mitogenomes used in the phylogenetic reconstruction. The complete mitogenome sequences used in the phylogenetic reconstruction of the family Saturniidae, including new and publicly available data as of November 2020. NA – information not available.

Species	Tribe	Distribution	Vernacular name	Specimen	Genbank	Reference	Size (bp)
Actias artemis aliena (Butler, 1879)	Saturniini	Japan, Korea, China, India, Malaysia	Japanese luna moth	NA	KF927042	Park *et al*, 2014	15,243
Actias luna (Linnaeus, 1758)	Saturniini	North America	Luna moth	NA	NC_045899	Langley *et al*, 2019	15,259
Actias selene (Hübner, 1807)	Saturniini	India, Japan, Nepal, Sri Lanka, Borneo and other Eastern Asian islands	Indian Luna moth	NA	NC_018133	Liu *et al*, 2012	15,236
Antheraea assama	Saturniini	Northeast India	Assam silk moth	NA	NC_030270	Direct submission	15,312
Antheraea assama	Saturniini	Northeast India	Assam silk moth	NA	KU301792	Direct submission	15,312
Antheraea assamensis (Helfer,1837)	Saturniini	Northeast India	Assam silk moth	NA	KU379695	Singh *et al* 2017	15,272
Antheraea frithi (Moore, 1859)	Saturniini	Northeast india	Wild oak tasar silkworm	IBSDIRWSA1	NC_027071	Shantibala *et al*, 2018	15,338
Antheraea pernyi (Guérin-Méneville, 1855)	Saturniini	Southern China, subtropical and tropical Asia	Chinese oak tasar silk moth	NA	NC_004622	Liu *et al*, 2008	15,566
Antheraea pernyi (Guérin-Méneville, 1855)	Saturniini	Southern China, subtropical and tropical Asia	Chinese oak tasar silk moth	Yu7	KP999979	Direct submission	15,572

Antheraea pernyi (Guérin-Méneville, 1855)	Saturniini	Southern China, subtropical and tropical Asia	Chinese oak tasar silk moth	NA	HQ264055	Liu *et al*, 2012	15,537
Antheraea pernyi (Guérin-Méneville, 1855)	Saturniini	Southern China, subtropical and tropical Asia	Chinese oak tasar silk moth	731	KP881616	In press	15,570
Antheraea pernyi (Guérin-Méneville, 1855)	Saturniini	Southern China, subtropical and tropical Asia	Chinese oak tasar silk moth	Yu6	KP762788	In press	15,569
Antheraea x *proylei* (hybrid A. pernyi x A. roley)	Saturniini	Southern China, subtropical and tropical Asia	Chinese oak tasar silk moth	In981	MK920216	Yang *et al*, 2019	15,573
Antheraea yamamai (Guérin-Ménéville, 1861)	Saturniini	East Asia and Europe	Japanese oak silk moth	NA	NC_012739	Kim *et al*, 2009	15,338
Attacus atlas (Linnaeus, 1758)	Attacini	Southeast Asia	Atlas moth	SYAU01	NC_021770	Chen *et al*, 2014	15,282
Bunaea alcinoe (Stroll, 1780)	Bunaeini	Southern Africa	Cabbage tree Emperor moth	BA1.6	OL912807	This study	15,305
Cricula trifesnestrata (Helfer,1837)	Saturniini	India to the Philippines, Sulawesi, Java, and Sri Lanka	Cricula silk moth	CMERI-Ct-001	KY644697	Direct submission	15,425
Epiphora bauhinia (Wallengren, 1860)	Attacini	Southern Africa	Southern Atlas moth	GM5.2	OL912809	This study	15,259
Eriogyna pyretorum (Westwood, 1847)	Saturniini	South and Eastern China, South Korea	Giant silkworm moth	NA	NC_012727	Jiang *et al*, 2009	15,327
Gonimbrasia belina (Westwood, 1894)	Bunaeini	Southern Africa	Emperor moth	F1Y	NC_046032	Langley *et al*, 2019	15,308

Gonimbrasia belina (Westwood, 1894)	Bunaeini	Southern Africa	Emperor moth	J6	MN832539	Langley *et al*, 2019	15,295
Gonimbrasia tyrrhea (Cramer, 1776)	Bunaeini	Southern Africa	Zigzag Emperor moth	GT1.1	OL912810	This study	15,299
Gynanisa maja (Klug, 1836)	Bunaeini	Southern Africa	Speckled Emperor moth	K5	NC_046033	Langley *et al*, 2019	15,299
Gynanisa maja (Klug, 1836)	Bunaeini	Southern Africa	Speckled Emperor moth	RA1	MN832541	Langley *et al*, 2019	15,287
Heniocha dyops (Maasen, 1886)	Bunaeini	Southern Africa	Western marbled Emperor moth	GM8.2	OL912812	This study	15,306
Neoris haraldi (Schawerda, 1922)	Saturniini	China	n.a.	NA	NC_036765	He *et al*, 2017	15,383
Nudaurelia cytherea (Fabricius, 1775)	Bunaeini	Southern Africa	Pine tree Emperor moth	NC1.2	OL912953	This study	15,363
Samia canningi (Hutton, 1859)	Attacini	South-eastern Asia, India, China	Wild eri silk moth	NA	NC_024270	Shantibala *et al*, 2014	15,384
Samia cynthia (Drury, 1773)	Attacini	China, Korea	Ailanthus silk moth	NA	KC812618	Sima *et al*, 2013	15,345
Samia cynthia ricini Boisd.	Attacini	China, Korea, India	Eri silk moth	NA	NC_017869	Kim *et al*, 2012	15,384
Samia cynthia ricini Boisd.	Attacini	China, Korea, India	Eri silk moth	NA	JF961379	Direct submission	15,420
Saturnia boisduvalii (Eversmann, 1847)	Saturniini	North and South Korea, Japan, Northern China, and Eastern Russia	Wild silk moth	SB4014	MF034742	Kim *et al*, 2017	15,257

Saturnia boisduvalii (Eversmann, 1849)	Saturniini	North and South Korea, Japan, Northern China, and Eastern Russia	Wild silk moth	NA	NC_010613	Hong *et al*. 2018	15,360
Saturnia jonasii (Butler, 1877)	Saturniini	Japan and Taiwan	n.a.	NA	MF346379	Kim *et al*, 2018	15,261
Vegetia ducalis (Jordan, 1922)	Micragonini	Southern Africa	Ducal princeling	VD1	OL912815	This study	15,218
Vegetia grimmia (Geyer, 1831)	Micragonini	Southern Africa	Grim princeling	VG2	OL912816	This study	15,253

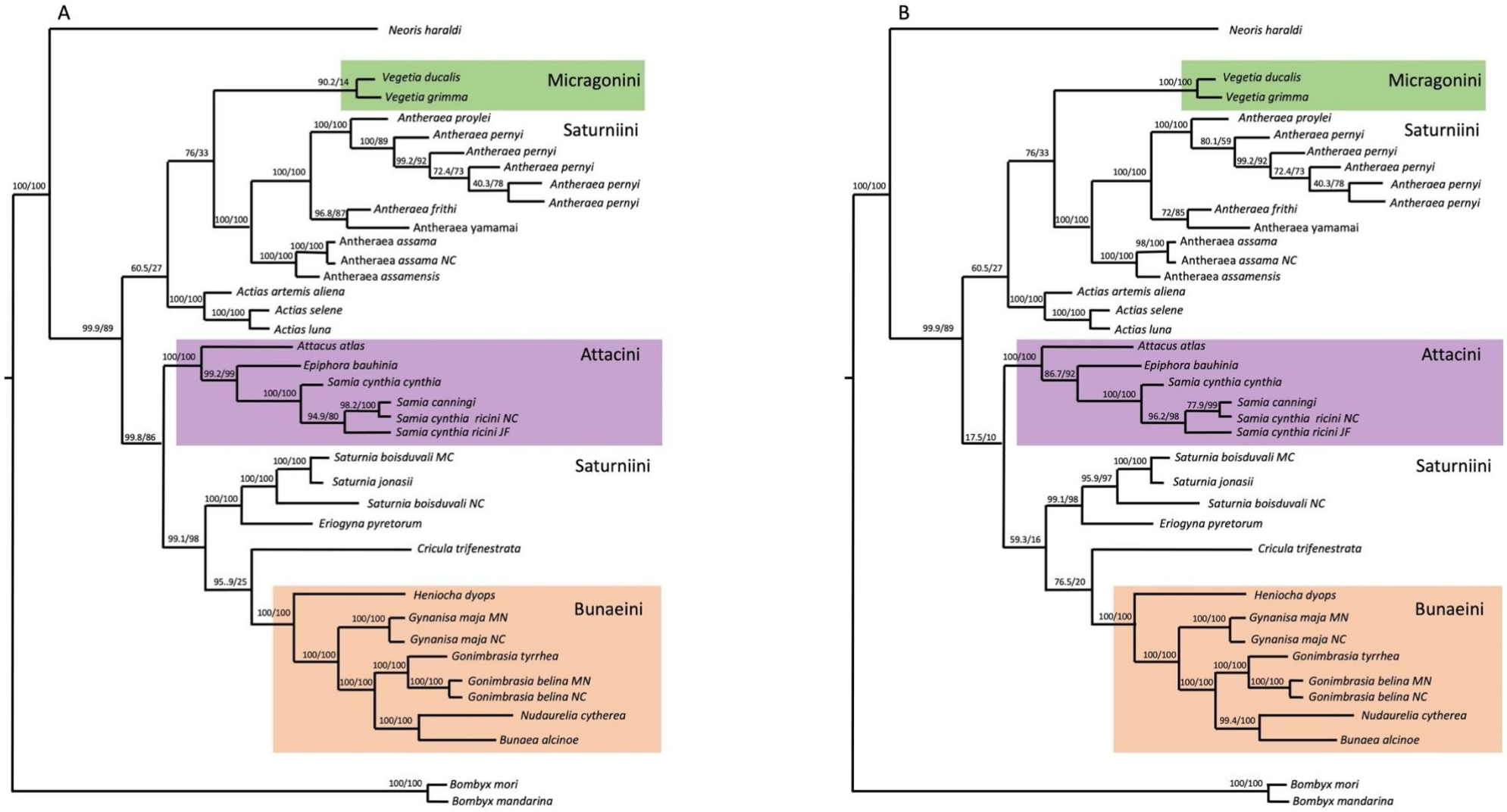

Figure S 1 PCG123 and PCG12 maximum likelihood phylogeny. Maximum likelihood reconstruction of the mitochondrial phylogeny of Saturniidae based on mitochondrial protein-coding genes (PCG). Nodal supports are shown as ultrafast bootstrap support (UFBoot)/approximate likelihood ratio test (SH-aLRT), at 1,000 replicates. (A) PCG12 (1st and 2nd codon positions). (B) PCG123 (all codon positions).

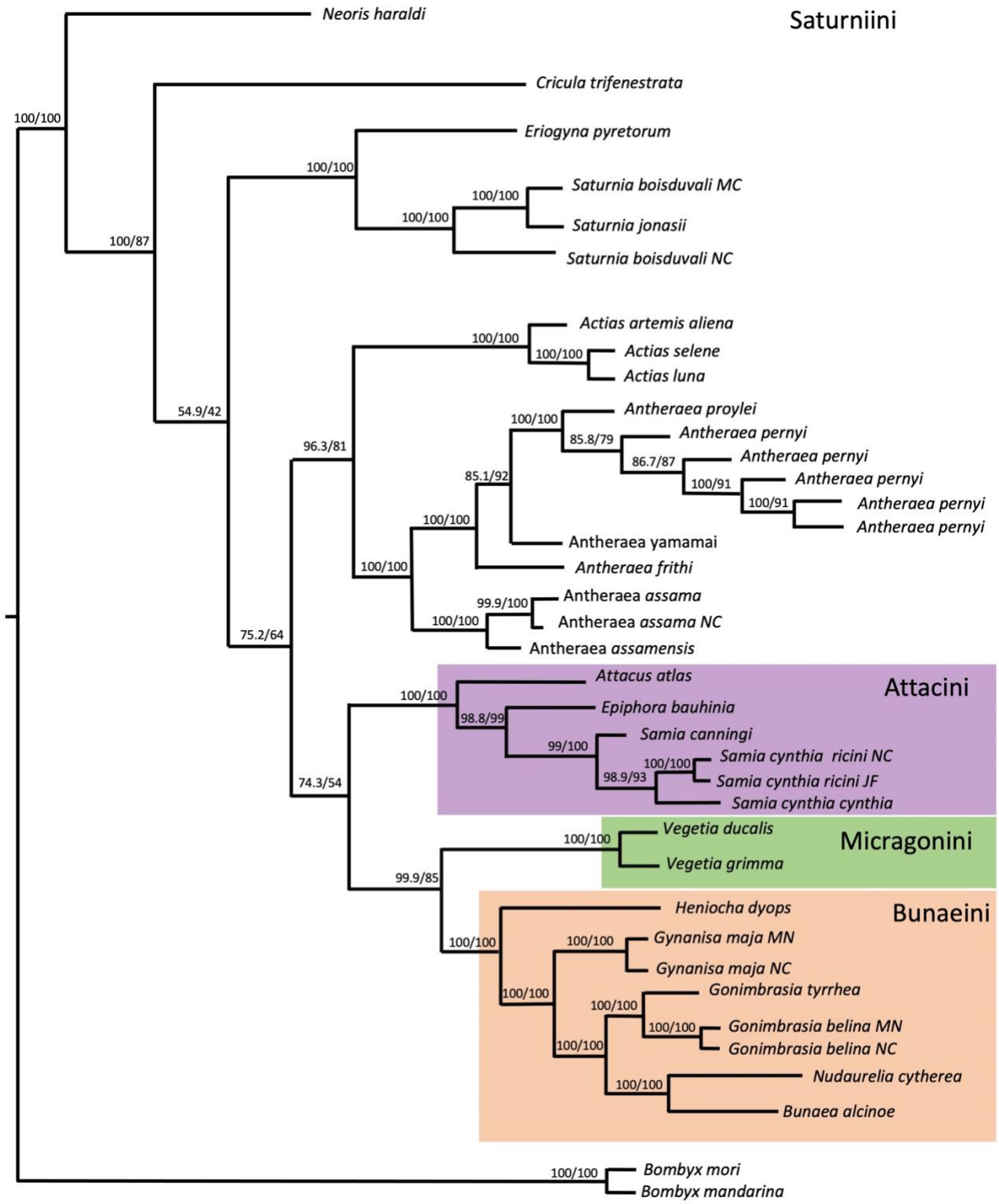

Figure S 2 PCG123+rRNA maximum likelihood phylogeny. Maximum likelihood reconstruction of the mitochondrial phylogeny of Saturniidae using all three codon positions of the total complement of 13 mitochondrial protein-coding genes and two rRNA genes (PCG123+rRNA). Nodal supports are shown as ultrafast bootstrap support (UFBoot)/approximate likelihood ratio test (SH-aLRT)at 1,000 replicates.

Table S3 Sample list. Sample list and information for *Gonimbrasia belina* collected in South Africa, Namibia and Botswana for this study.

Specimen	Country	Collection	Collection site	Longitude	Latitude
D3	Botswana	31 March 2021	Dikabeya	-21.59290	27.12335
D7	Botswana	31 March 2021	Dikabeya	-21.59290	27.12335
L12	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L13	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L14	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L17	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L18	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L20	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L21	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L22	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L24	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L27	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L29	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L36	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L40	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L41	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L46	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L48	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L5	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L6	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
L8	Botswana	01 April 2021	Letlapeng	-22.00592	27.14462
Mj11	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj13	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj14	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj16	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj17	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj18	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj25	Botswana	01 April 2021	Majojo	-21.59290	27.12335
Mj27	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj3	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj4	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj43	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mj5	Botswana	01 April 2021	Majojo	-21.59290	27.17196
Mo2	Botswana	01 April 2021	Moremi	-22.33510	27.26200
P8	Botswana	01 April 2021	Palapye	-22.29370	27.08286
S1	Botswana	01 April 2021	Serule	-21.55250	27.19191
S7	Botswana	01 April 2021	Serule	-21.55250	27.19191
T12	Botswana	01 April 2021	Topisi	-22.16345	27.14471
T3	Botswana	01 April 2021	Topisi	-22.16345	27.14471
T5	Botswana	01 April 2021	Topisi	-22.16345	27.14471
T6	Botswana	01 April 2021	Topisi	-22.16345	27.14471
T8	Botswana	01 April 2021	Topisi	-22.16345	27.14471

T9	Botswana	01 April 2021	Topisi	-22.16345	27.14471
Uk13	Botswana	01 April 2021	Unknown	-21.50202	27.20310
Uk15	Botswana	01 April 2021	Unknown	-21.50202	27.20310
Uk16	Botswana	01 April 2021	Unknown	-21.50202	27.20310
Uk2	Botswana	01 April 2021	Unknown	-21.50202	27.20310
Uk23	Botswana	01 April 2021	Unknown	-21.50202	27.20310
Uk33	Botswana	01 April 2021	Unknown	-21.50202	27.20310
Uk4	Botswana	01 April 2021	Unknown	-21.50202	27.20310
Uk7	Botswana	01 April 2021	Unknown	-21.50202	27.20310
A01	South Africa	21 March 2018	Close to Platjan lodge	-22.48702	28.80892
A02	South Africa	21 March 2018	Close to Platjan lodge	-22.48697	28.80892
A03	South Africa	21 March 2018	Close to Platjan lodge	-22.48672	28.80930
A04	South Africa	21 March 2018	Close to Platjan lodge	-22.48695	28.80927
A05	South Africa	21 March 2018	Close to Platjan lodge	-22.48705	28.80958
A06	South Africa	21 March 2018	Close to Platjan lodge	-22.48720	28.80983
A07	South Africa	22 March 2018	Limpopo	-22.52242	28.82620
A08	South Africa	22 March 2018	Limpopo	-22.48122	28.83892
A09	South Africa	22 March 2018	Limpopo	-22.50165	28.87262
A10	South Africa	22 March 2018	Limpopo	-22.48935	28.87277
A11	South Africa	22 March 2018	Limpopo	-22.48122	28.83722
A12	South Africa	22 March 2018	Limpopo	-22.52237	28.82238
A13	South Africa	22 March 2018	Limpopo	-22.50163	28.87263
A14	South Africa	22 March 2018	Limpopo	-22.48177	28.83763
A15	South Africa	22 March 2018	Limpopo	-22.50465	28.87080
A16	South Africa	22 March 2018	Limpopo	-22.48035	28.87092
A17	South Africa	22 March 2018	Limpopo	-22.50427	28.85918
A18	South Africa	22 March 2018	Limpopo	-22.50332	28.80573
B01	South Africa	22 March 2018	Limpopo	-22.50413	28.85917
C01	South Africa	21 March 2018	Close to Platjan lodge	-22.50402	28.80843
C04	South Africa	22 March 2018	Limpopo	-22.50402	28.87085
D01	South Africa	21 March 2018	Close to Platjan lodge	-22.50403	28.80910
D02	South Africa	22 March 2018	Limpopo	-22.50403	28.80645
E01	South Africa	22 March 2018	Limpopo	-22.50403	28.80813
F01	South Africa	21 March 2018	Close to Platjan lodge	-22.50403	28.80908
G02	South Africa	22 March 2018	Limpopo	-22.504040	28.82238
G03	South Africa	22 March 2018	Limpopo	-22.504040	28.82115
GI01	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI02	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI03	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI04	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI05	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI06	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI07	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI08	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI09	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439

GI10	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI11	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI12	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI13	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI14	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI15	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
GI20	South Africa	29 December 2018	Nkomo village	-23.407247	30.78439
H01	South Africa	21 March 2018	Close to Platjan border	-22.504040	28.80747
H02	South Africa	22 March 2018	Limpopo	-22.504040	28.83910
H03	South Africa	22 March 2018	Limpopo	-22.504040	28.85145
H06	South Africa	22 March 2018	Limpopo	-22.504040	28.87108
H08	South Africa	22 March 2018	Limpopo	-22.504040	28.82030
I02	South Africa	21 March 2018	Close to Platjan lodge	-22.504040	28.80908
MA01	South Africa	31 December 2018	Mapesu Nature reserve	-22.249243	29.46751
MA02	South Africa	31 December 2018	Mapesu Nature reserve	-22.249243	29.46751
MA03	South Africa	31 December 2018	Mapesu Nature reserve	-22.249243	29.46751
MA05	South Africa	31 December 2018	Mapesu Nature reserve	-22.249243	29.46751
MP01	South Africa	30 December 2018	Mapungubwe R572	-22.243065	29.39031
MP06	South Africa	30 December 2018	Mapungubwe R572	-22.243065	29.39031
MP11	South Africa	30 December 2018	Mapungubwe R572	-22.243065	29.39031
GM4.2	Namibia	09 March 2019	AfriCat North House	-19.476274	14.59663
J05	Namibia	14 March 2017	Windpoort farm	-19.341430	15.45380
J06	Namibia	14 March 2017	Windpoort farm	-19.341430	15.45380
J09	Namibia	14 March 2017	Windpoort farm	-19.341430	15.45380
J11	Namibia	14 March 2017	Windpoort farm	-19.341430	15.45380
J13	Namibia	14 March 2017	Windpoort farm	-19.34143	15.45380
MH2	Namibia	22 December 2020	AfriCat North House	-19.475121	14.59458
MH6	Namibia	23 December 2021	AfriCat North House	-19.475121	14.59458

Table S4 Average nucleotide base composition. Average nucleotide base composition of *Gonimbrasia belina* specimens collected in Botswana, South Africa and Namibia based on concatenated COI and amplicons A (ATP6/COIII) and C (ND6/CYTB) sequence data.

Country	**A%**	**T%**	**C%**	**G%**	**AT content %**	**CG content %**
Botswana	33.81	40.22	14.80	11.17	74.03	25.97
South Africa	34.05	40.22	14.70	11.03	74.27	25.73
Namibia	33.74	40.46	14.40	11.40	74.20	25.80

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