Species identification, lifestyles and mitogenomics of the elusive wasp assemblage associated with wild and cultivated olives in the Western Cape

By

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Declaration

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This thesis includes one original paper published in a peer-reviewed journal. The development and writing of the paper were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Date: December 2019

Abstract

While the international olive industry is greatly affected by the olive fruit fly (*Bactrocera oleae*), South African commercial crops are marginally affected. The rich assemblage of native parasitoid wasps found in the region may contribute to maintain *B. oleae* populations at low levels and has potential applications in biocontrol. Furthermore, agricultural losses in the Western Cape province are mainly due to the activity of olive seed wasps (OSW) associated with premature fruit drop; however, the status of several species as phytophagous, parasitoid and hyperparasitoid remains uncertain. Moreover, the genus *Psyttalia* (Braconidae: Opiinae) includes parasitoid species with potential for biocontrol of fruit-infesting tephritid pests such as *B. oleae*. In addition to the native Mediterranean tephritid parasitoid, *Psyttalia concolor*, two sub-Saharan parasitoid species; *Psyttalia humilis* and *Psyttalia lounsburyi*, are of particular interest as they can be utilized in exotic biocontrol applications of *B. oleae.*

The aim of this study was to generate a comprehensive catalogue of the entomofauna associated with olive fruit by identifying all species affecting the fruit, confirming the status of putative olive seed wasps as phytophagous and contributing to the phylogeny of the family Braconidae. The comprehensive survey combined morphological and DNA-based methods in which four Braconidae species and six Chalcidoidea species were identified. Furthermore, morphological identifications were congruent with DNA data for all species, as neighbour-joining and maximum-likelihood trees correctly placed all sequences either at the genus or species level, depending on the available taxa coverage, and low genetic distances strongly supported conspecificity. These results can be used for early DNA-based species identification of wasp larvae in olives, and to further investigate their biology and ecology.

In addition, a DNA-based identification tool was developed to identify immature specimens collected from olive seeds and for the simultaneous detection of five putative chalcid OSW (*Eupelmus spermophilus, Eurytoma oleae, Eurytoma varicolor, Neochrysocharis formosus* and *Sycophila aethiopica*). *Eupelmus spermophilus*, *E. oleae* and *S. aethiopica* were identified in olive seeds, and confirmed as phytophagous. This method can be used as a diagnostic tool for pest detection at an early stage of infestation when morphological identification is not feasible. Furthermore, it can provide new biological insights into the lifestyles of these elusive species.

The near-complete mitochondrial genomes of *Psyttalia concolor, Psyttalia humilis,* and *Psyttalia lounsburyi,* three important parasitoids, were recovered using Sanger sequencing and next-generation sequencing. The three *Psyttalia* species had a particular gene arrangement involving tRNAs, different from all other Braconidae. Gene rearrangements varied substantially among Braconidae, potentially being useful for clarification of the complex phylogeny of the group. Phylogenetic reconstruction within the family Braconidae confirmed the division of two major lineages of cyclostome and non-cyclostome braconids. Furthermore, our analyses corroborated subfamily Aphidiinae as a sister group to the cyclostome braconids, a group previously thought to be a separate family (Aphidiidae). Overall, this study advances the knowledge of insects associated with olive fruit in the Western Cape, the status of phytophagous OSW, which was previously undetermined, and contributes to the phylogeny and understanding of gene rearrangement in the family Braconidae.

Opsomming

Terwyl die internasionale olyf-industrie sterk beïnvloed word deur die olyf-vrugtevlieg (*Bactrocera oleae*), word die Suid-Afrikaanse kommersiële bedryf gering geraak deur die pesinsek. Die diverse samestelling van inheemse parasitiese wespe wat in die streek voorkom, mag bydra tot die beheer van *B. oleae*-populasies en vlieg-getalle laag hou en het dus potensiële toepassings in biobeheer. Verder is landbouverliese in die Wes-Kaap provinsie hoofsaaklik te wyte aan die aktiwiteit van olyfsaadwespe (OSW) wat verband hou met voortydige vrugval. Die status van verskillende olyfsaadwespe spesies as fitofagus, parasitoïed en hiperparasitoïed bly egter onseker. Die genus *Psyttalia* (Braconidae: Opiinae) bevat ook parasitiese spesies met die potensiaal vir biobeheer van vrugtebesmette tephritiede soos *B. oleae*. Benewens die inheemse Mediterreense tephritid-parasitoïed, is *Psyttalia concolor*, twee sub-Sahara-parasitoid-spesies; *Psyttalia humilis* en *Psyttalia lounsburyi* van besondere belang, aangesien dit gebruik kan word in eksotiese bio-beheertoepassings van *B. oleae*.

Die doel van hierdie studie was om 'n omvattende katalogus van die entomofauna geassosieer met olyfvrugte saam te stel, deur spesies wat die vrug beïnvloed te identifiseer; om die vermeende status van die olyfsaadwesp as fitofagus te bevestig; en by te dra tot die filogenie van die familie Braconidae. Die uitgebreide opname het morfologiese en DNS-gebaseerde metodes gekombineer waarin vier Braconidae-spesies en ses Chalcidoidea-spesies geïdentifiseer is. Verder was morfologiese identifikasies ooreenstemmend met DNS-data vir alle spesies, met “neighbour-joining” en “maximum-likelihood” bome wat DNS volgordes in die korrekte genus- of spesies-kategorie geplaas het, afhangend van die beskikbare taxa-dekking, en genetiese afstande ondersteun ook die gevolgtrekking. Hierdie resultate kan gebruik word om vroeë DNS-gebaseerde spesie identifikasie van wesplarwes in olywe te doen, en om hul biologie en ekologie verder te ondersoek.

Daarbenewens, is 'n DNS-gebaseerde identifikasiehulpmiddel ontwikkel om onvolwasse eksemplare te identifiseer wat versamel is uit olyfsade vir vyf vermeende chalcied OSW (*Eupelmus spermophilus*, *Eurytoma oleae*, *Eurytoma varicolor*, *Neochrysocharis formosus* en *Sycophila aethiopica*). *Eupelmus spermophilus*, *E. oleae* en *S. aethiopica* is in olyfsade geïdentifiseer en as fitofagus bevestig. Hierdie metode kan gebruik word as 'n diagnostiese hulpmiddel vir die opsporing van plae in 'n vroeë stadium van infestasie wanneer

morfologiese identifikasie nie haalbaar is nie. Verder bied dit nuwe biologiese insigte oor die lewensstyle van hierdie ontwykende spesies.

Die byna volledige mitochondriale genoom van *Psyttalia concolor*, *Psyttalia humilis*, en *Psyttalia lounsburyi*, drie belangrike parasitoïede, is met behulp van die Sanger en volgende generasie volgordebepaling bepaal. Die drie *Psyttalia*-spesies het 'n spesifieke gene-rangskikking gehad wat tRNA's behels, wat anders as alle ander Braconidae is. Geenherrangskikking wissel aansienlik onder Braconidae, wat nuttig kan wees om die komplekse filogenie van die groep te ontbloot. Filogenetiese rekonstruksie binne die familie Braconidae het die monofilie van twee hoofstamlyne van siklostoom- en nie-siklostoombrakoniede bevestig. Verder het die ontleding die subfamilie Aphidiinae bevestig as 'n sustergroep vir die siklostome-braconiede, wat voorheen as 'n afsonderlike familie (Aphidiidae) beskou was. In die algemeen bevorder hierdie studie die kennis van insekte wat in die Wes-Kaap geassosieer word met olyfvrugte, die status van fitofagiese OSW, wat voorheen onbepaald was, en dra dit by tot die filogenie en die begrip van geen-herrangskikking in die familie Braconidae.

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Abbreviations

% - Percentage

® - Registered Trademark

™ - Trademark

°C – Degrees Celsius

3´ - Three prime

5´ - Five prime

ATP6 – Adenine triphosphate synthase membrane subunit 6

ATP8 – Adenine triphosphate synthase membrane subunit 8

APR – Apparent parasitism rate

Bp – Base pairs

BLAST – Basic Local Alignment Search Tool

BOLD – Barcode of Life Database

CIPRES – Cyberinfrastructure for Phylogenetic Research

COI – Cytochrome oxidase subunit 1

COII – Cytochrome oxidase subunit 2

COIII – Cytochrome oxidase subunit 3

COX1 – Cytochrome oxidase subunit 1

COX2 – Cytochrome oxidase subunit 2

CYTB – Cytochrome b

cpDNA – Chloroplast deoxyribonucleic acid

DNA – Deoxyribonucleic acid

DTI – Department of Trade and Industry

EU – European Union

F – Forward primer

GTR – General Time Reversible model

IF – Infestation rate

IOOC – International Olive Oil Council

ITS – Internal transcribed spacer

K2P – Kimura-2-parameter

Ltd - Limited

MAFFT – Multiple alignment using fast Fourier transform

MEGA7 – Molecular Evolutionary Genetic Analysis 7

$MgCl_2$ – Magnesium Chloride

min – Minutes

mm – Millimetre(s)

mM – Milimolar

mL – Millilitre(s)

ML – Maximum-likelihood

mtDNA – Mitochondrial deoxyribonucleic acid

MYA – Million years ago

$(NH_4)_2SO_4$ – Ammonium sulphate

NADH – Nicotinamide adenine dinucleotide + hydrogen

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

NGS – Next-generation sequencing

NSW –New South Wales

NUMTs – Nuclear pseudogenes of mitochondrial origin

OSW – Olive seed wasp

PAROFFIT – Parasitoid of Fruit-infesting Tephritidae

PCGs – Protein-coding genes

PCR – Polymerase chain reaction

pmol - Picomole

RFLP – Restriction fragment length polymorphism

RNA – Ribonucleic acid

rRNA – Ribosomal ribonucleic acid

R – Reverse primer

s – Seconds

RSA – Republic of South Africa

SAOGA – South African Olive Growers' Association

SA Olive – South African Olive Association

tRNA – Transfer ribonucleic acid

U – Units (enzyme)

µM – Micromolar

µL – Microlitre(s)

v – Version

v/v – Volume per volume

Chapter 1: Study overview, rationale and outcomes

1.1 General introduction

For centuries, the olive tree has been used as a source of food, oil, wood, cattle fodder, and in rituals and religious practices (Besnard *et al.*, 2018; Diez *et al.*, 2015). As a result, the olive tree has become an iconic species of the Mediterranean Basin (Kaniewski *et al.*, 2012; Zohary *et al.*, 2012). Today, numerous cultivars are grown for the production of high-quality olive oil and table olives. According to the International Olive Oil Council, the 2016/2017 crop year for olive oil production, recorded 2,561,500 tonnes, which substantially increase in 2017/2018, where 3,314,500 tonnes were recorded worldwide (IOOC, 2019). Moreover, the 2016/2017 crop year recorded 2,899,500 tonnes of olives destined for table olive production, whereas in 2017/2018, a slight increase was observed with 2,908,500 tonnes reported worldwide (IOOC, 2019).

The olive industry in South Africa is relatively new, and despite the recent increase in olive oil and table olive production, the country remains a minor player in the industry compared to regions experiencing increasing cultivation such as Argentina, Australia, Chile, China and the United States (Diez *et al.*, 2015). In spite of the low quantity produced, South African olive oil has high organoleptic quality and has recently won several awards, for example, multiple gold medal award winners included Babylonstoren, De Rustica Estate, Morgenster, Oakhurst Olives, and Porterville Olives. In 2019, 81 locally produced extra-virgin olive oil received medals (25 gold, 35 silver, and 21 bronze medals) at the South African Olive Association award ceremony, judged by international and local experts (SA Olive, 2019).

Agricultural losses in olive production attributed to biological agents have been reported to be as high as 30%, and caused by insect pests, weeds and fungi. Insect pests causing damage to fruit are responsible for an estimated 15% (Bueno & Jones, 2002). *Bactrocera* species belonging to the family Tephritidae (“true fruit flies”) are among the most significant pests of fruits and vegetables (Clarke *et al.*, 2005). *Bactrocera oleae* (Rossi) is the most economically damaging agricultural pest of olive-producing countries worldwide. The distribution of the olive fruit fly includes most regions where the cultivated olives are extensively produced and wild olives occur naturally (Nardi *et al.*, 2005). Furthermore, the olive fruit fly seems to have the ability to rapidly colonize new environments suggesting that all countries growing olives are at risk of invasion.

As such, much attention has focused on finding effective control management methods of the olive fruit fly. For over a century, death or chemosterilization was achieved by the use of baits containing attractive compounds. In the last four decades, olive fruit fly populations are maintained at low levels primarily by the use of insecticides, such as organophosphates (Daane & Johnson, 2010). However, insecticide resistance and the detrimental environmental effects of insecticides has given relevance to the search for biological control measures. Several European programs for the control of the olive fruit fly focused on the release of *Psyttalia concolor* (Szépligeti), a parasitoid wasp attacking the larvae of the olive fruit fly in wild and cultivated olives in the Mediterranean region. Additionally, parasitoid species native to sub-Saharan Africa, such as *Psyttalia humilis* and *Psyttalia lounsburyi*, have recently been tested in California; however, their release was unsuccessful. Therefore, understanding the diversity of parasitoid species and their biology is imperative for development of biological control of the olive fruit fly.

In South Africa, the olive fruit fly is not regarded as an agricultural pest as infestation levels do not reach economic damaging levels. However, production losses due to insect pests occur, and are mainly a consequence of the activity of olive seed wasps (OSW) that cause premature fruit drop. Earlier surveys have reported on the rich diversity of parasitoids and seed wasps in the Western Cape, and since then more entomological surveys have been conducted; yet, no molecular classification studies have been done. As such, it is essential to identify parasitoids for potential biocontrol and to verify olive seed wasp species as the current status of several species remains undetermined. Identification of species at the immature stage *i.e.* larvae and pupae, could aid in the detection of early infestations of OSW and inform strategies for control.

The focus of this study was to contribute to the existing knowledge of the wasp assemblage associated with olive fruit in the Western Cape province including elucidating lifestyles and contributing to the phylogeny of the family Braconidae. This study has implications at a global scale, as sub-Saharan parasitoid species hold the potential to serve as biological control agents of olive fruit fly in world areas where this pest is significantly damaging. This study also has implications at the regional scale, as early identification and detection of OSW infestation could aid in preventative management.

1.2 Aims and objectives

The study aimed to generate a comprehensive catalogue of the entomofauna associated with wild and cultivated olives by identifying all species affecting the fruit in the Western Cape province of South Africa, confirming the status of putative olive seed wasps as phytophagous and contributing to the phylogeny of the family Braconidae. The following objectives were set out to achieve this aim:

A) To use an integrated approach of morphological identification and DNA barcoding of wasps associated with wild and cultivated olives in the Western Cape
B) To elucidate the lifestyles of putative olive seed wasp species for which the status is presently unknown, using reference DNA sequences generated in the previous objective
C) To contribute to the elucidation of the phylogeny of the family Braconidae, an important parasitoid wasp family specialized in the olive fruit fly.

1.3 Chapter layout

This thesis is divided into six chapters: an introduction, literature review, three research chapters and a conclusion. The references of across all chapters are given at the end of the document.

Chapter 1: Introduction

A general introduction of the field, the study and its significance, aims and objectives, an overview of the layout of the chapters, and the research outputs generated throughout the study are provided.

Chapter 2: Literature review

An overview of the literature regarding the origin, domestication, and dissemination of olives, the importance of table olives and olive oil, and the insects associated with wild and cultivated olives.

Chanter 3: Barcoding of parasitoid wasps (Braconidae and Chalcidoidea) *associated with wild and cultivated olives in the Western Cape*

A comprehensive survey performed for the cataloguing of insects associated with wild and cultivated olives in the Western Cape is described. An integrated approach of morphological- and molecular-based species identification methods to identify wasp species was used.

Chapter 4: DNA-based species identification of insect larvae gives new insight into the elusive lifestyles of olive seed wasps

The development and application of a multiplex PCR method for the DNA-based species identification of immature insect specimens collected from within the seed structure of wild and cultivated olives in the Western Cape province is described.

Chapter 5: The mitochondrial genomes of Psyttalia concolor, Psyttalia humilis and Psyttalia lounsburyi, three parasitoid wasps of the olive fruit fly Bactrocera oleae

The partial mitochondrial genomes of three parasitoid wasps, as determined by NGS, are presented. The diversity of gene rearrangements within Braconidae are discussed. Phylogenetic reconstruction was used to infer evolutionary relationships within the family Braconidae.

Chapter 6: Conclusion

General concluding remarks, limitations and future prospects of the study.

1.4 Research outputs

The following publication and conference contributions were generated during the study:

Article in international peer-reviewed journal

Powell, C., Caleca, V., Sinno, M., van Staden, M., van Noort, S., Rhode, C., Allsopp, E., van Asch, B. 2019. Barcoding of parasitoid wasps (Braconidae and Chalcidoidea) associated with wild and cultivated olives in the Western Cape of South Africa. *Genome*, 62(3), pp. 183–199. doi: 10.1139/gen-2018-0068.

This publication forms part of Chapter 3. The supplementary data can be found online at: https://doi.org/10.1139/gen-2018-0068

Conference contributions

Powell, C., Caleca, V., Sinno, M., van Staden, M., van Noort, S., Rhode, C., Allsopp, E., van Asch, B. Barcoding of parasitoid wasps (Braconidae and Chalcidoidea) associated with wild and cultivated olives in the Western Cape province of South Africa. The Joint Biodiversity Information Management and Foundational Biodiversity Information Programme (BIMF-FBIP) Forum. Cape St Francis, South Africa. August 13 – August 16, 2018.

Oral presentation summarising the research performed in Chapter 3, presented by C. Powell

Powell, C., Knipe, M., Caleca, V., Allsopp, E., van Noort., Langley, J., van Asch, B. A multiplex PCR test for species identification of elusive wasps associated with olives in South Africa. Entomological Society of Southern Africa 21st Congress. Durban, South Africa. July 08 – July 11, 2019.

Poster presentation summarizing research performed in Chapter 4, presented by C. Powell

Powell, C., Caleca, V., Allsopp, E., van Noort, S., Langley, J., Teixeira da Costa, L., van Asch, B. The mitochondrial genomes of two important parasitoids of olive fruit lies in South Africa (*Psyttalia humilis* and *Psyttalia lounsburyi*) show a new gene rearrangement among Braconidae. Entomological Society of Southern Africa 21st Congress. Durban, South Africa. July 08 – July 11, 2019.

Poster presentation summarizing the results of Chapter 5, presented by C. Powell.

Chapter 2: Literature review

2.1 Introduction

The olive (*Olea europaea* L.) is a small tree belonging to the family Oleaceae, which comprises of 24 genera and roughly 600 species (Besnard *et al.*, 2009; Parvaiz *et al.*, 2013). Within the tribe *Oleeae*, *Olea* and ten other extant genera constitute the subtribe *Oleinae* (Besnard *et al.*, 2009). The genus *Olea* L. consists of *Olea europaea* L. as the only cultivated species. Two forms of this species co-exist including the wild olive, or oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris* (Miller) Lehr.), and the cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*). The genus *Olea* L. exclusively comprises the *Olea europaea* complex, in which six subspecies are recognized on the basis of morphological classification: (a) subspecies *europaea* present in the Mediterranean basin; (b) subspecies *cuspidata* (Wall ex. G. Don) Cif., which is commonly known as the African olive, distributed from South and East Africa, to Southeast Asia, Southwest China, and Arabia; (c) subspecies *laperrinei* (Batt. & Trab.) Cif. present in Saharan mountains; (d) subspecies *maroccana* Greut. & Burd. present in Southwestern Morocco; (e) subspecies *cerasiformis* (Webb & Berth.) Kunk. & Sund. present in Madeira; and (f) subspecies *guanchica* P. present in the Canary Islands (Green, 2002; Vargas, Muñoz, Hess & Kadereit, 2001). *Olea europaea* subspecies are diploid except for *maroccana* and *cerasiformis*, which are polyploid (Besnard *et al.*, 2018). These subspecies are thought to be inter-fertile with the Mediterranean olive (Besnard *et al.*, 2001), and gene flow between distant populations may have contributed to the evolution of the olive (Quezel, 1978). *Olea europaea* is also found outside its native distribution as a result of human-mediated dispersal, and has become naturalized and invaded regions such as Australia, New Zealand and the Pacific Islands (Besnard *et al.*, 2007; Green, 2002). Many cultivars are presently available worldwide, with the most common including Mission, Kalamata, Manzanilla, Barouni and Frantoio (Costa, 1998). Mission is the most common olive cultivar in the Western Cape due to its suitability for processing both table olives and olive oil.

2.2 Global production of olive oil and table olives

Olive trees can withstand unfavourable conditions, but in order to flourish, they are a demanding crop that requires a suitable environment and proper management necessary for the full development of agronomic traits and steady production (Uylaşer & Yildiz, 2014). Presently, the olive tree is cultivated in many countries including Spain, Greece, Italy, Portugal, Tunisia, Turkey, Morocco, Syria, Algeria, Egypt, Israel, Libya, Cyprus, Croatia, Jordan, Lebanon, Slovenia, Argentina, Chile, Mexico, Peru, the United States of America, and Australia (Boskou, 2009). More than 800 million olive trees are presently grown worldwide with more than 90% grown for olive oil production and the remaining 10% for table olives. The European Union (specifically, Spain, Greece, Italy, Portugal and France) is the largest contributor to total olive production (81%), followed by North Africa (~11%) and the Near-East (~7%). and Argentina, Mexico, Peru and the United States contribute with the remaining 1% (Uylaşer & Yildiz, 2014). According to the report of the International Olive Oil Council (IOOC, 2019), the worldwide olive oil production was estimated at 3,131,000 tonnes with the European Union (EU) (Spain, France, Greece, Italy, Portugal and Slovenia) contributing 2,219,000 tonnes (~ 70%) and other countries with significant contributions included Morocco (6.4%), Turkey (5.8%), Tunisia (3.8%), and Syria (3.2%) (Figure 2.1a) (IOOC, 2019). The worldwide production of table olives was estimated at 2,751,500 tonnes, chiefly produced by the EU (~ 31%) while other countries producing a significant amount included Egypt (16.4%), Turkey (15.3%), Algeria (12.5%), Morocco (4.7%) and Syria (3.6%) (Figure 2.1b).

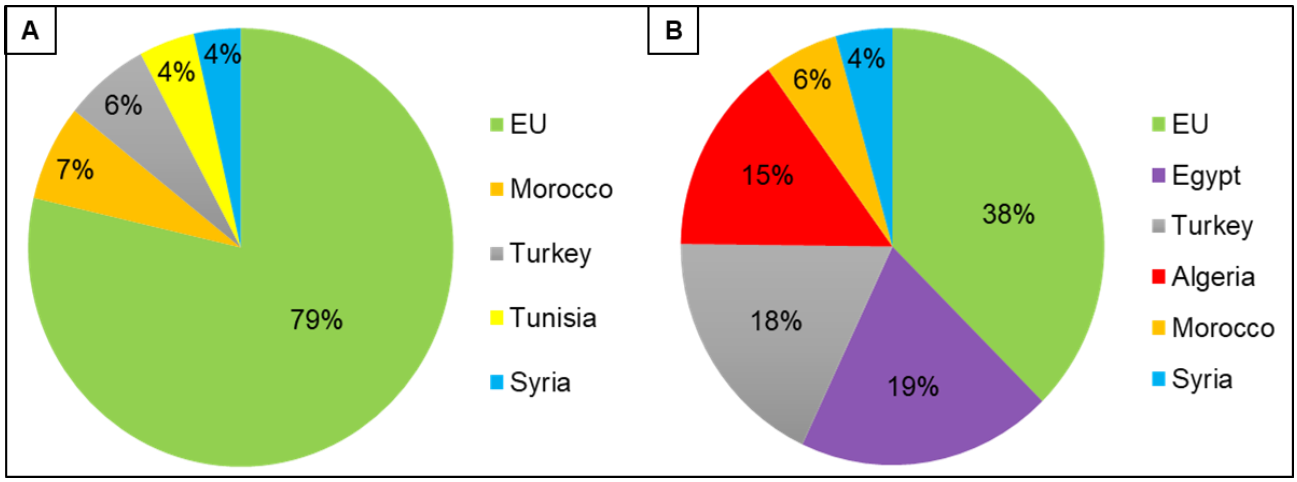

Figure 2.1. Olive oil and table olive production in 2018 - 2019 (IOOC). (A) Worldwide production of olive oil. (B) Countries with significant table olive production.

2.3 Production of olive oil and table olives in South Africa

Despite the arrival of olive trees in the Western Cape province of RSA during the late 17th century, the olive industry only started gaining momentum in the 1970's. Several factors contributed to the rapid expansion of the South African olive industry, including the establishment of the SAOGA, increased research, more awareness of the health benefits of consuming olive products, and improved olive marketing (Costa, 1998). Nevertheless, RSA remains a minor contributor to worldwide production of olives, and the rank of with regards to the rest of the world is undetermined. The olive sector development plan (2013) of the Department of Trade and Industry (DTI) estimated local olive oil production around 2,000 tons or 2 million litres, which translates into 10,000 tons of table olives. Furthermore, according to the annual report of the chairperson of SA Olive Nick Wilkinson, a decrease in production of olive oil was observed after 2016 attributed to the drought experienced resulting in 1288 tons and 1380 tons produced in 2017 and 2018 respectively, as opposed to the 202,4 tons produced in 2016 (Figure 2.2).

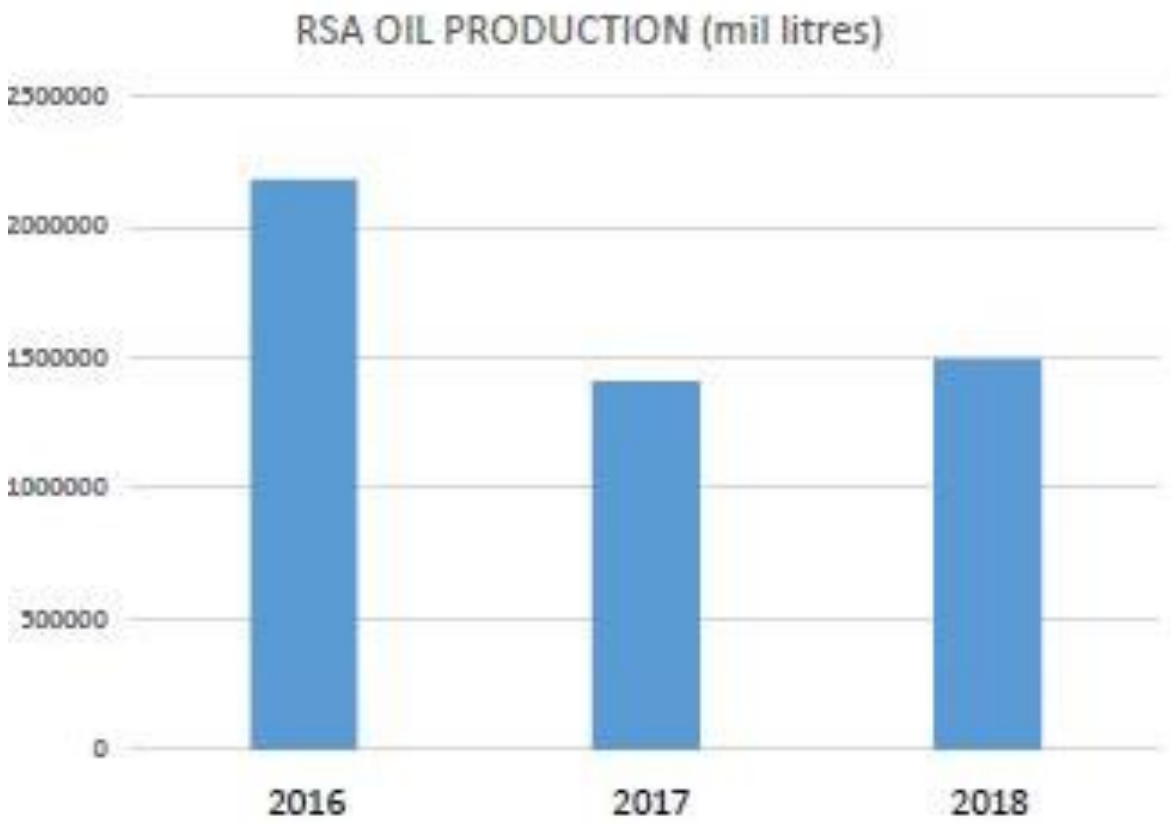

Figure 2.2. Republic of South African olive oil production in 2016, 2017, and 2018 (million litres) according to SA Olive annual report (Chairperson, Nick Wilkinson 2019).

2.4 Origin and dissemination of olives

The knowledge of genetic diversity in fruit trees is relevant for the management and improvement of agricultural resources. As such, characterizing the genetic diversity of cultivated plants and their wild ancestors is vital for investigating similarities and determining potential origins of cultivars (Besnard *et al.*, 2001). Furthermore, this approach provides insight into processes of domestication, possible hybridization events (or introgression) and demonstrate the influence of humans in the dispersal of cultivated olives (Besnard & Bervillé, 2000). In several fruit tree species, the analysis of morphological data suggested that hybridization between cultivars and their wild relatives contributed to the improvement in terms of agronomic/production traits and by implication the diversification of cultivars (Zohary, 1994). Several molecular studies have supported hybridization events in apple, cacao and coffee (Durham & Korban, 1994; Kato *et al.*, 1992; Lashermes *et al.*, 1999; Lerceteau *et al.*, 1997).

The cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) is a symbolic Mediterranean olive tree that is associated with the Mediterranean civilization. For centuries, olives have been used for food and oil, rituals and religion. Today, the olive remains an essential crop due to its economic importance worldwide, producing high-quality fruit for human consumption as olive oil and table olive (Bartolini *et al.*, 1998; Diez *et al.*, 2015). In spite of its economic, cultural and historical significance, the history of the olive remains mysterious and hotly debated. The wild Mediterranean olive, also referred to as oleaster (*Olea europaea* subsp. *europaea* var. *sylvestris* (Miller) Lehr.), has been thought to serve as a genetic reservoir from which the present cultivars originated (Zohary & Spiegel-Roy, 1975). Moreover, as individual trees are able to survive for long periods of time that may extend over 1,000 years, it is presumed that wild olives could in fact be ancient cultivars; therefore, some genotypes relating to very ancient cultivars may still be present (Besnard *et al.*, 2002).

2.4.1 Human-mediated dispersal

Fossil and sub-fossil records indicated that wild olive tree populations re-colonized the Mediterranean area from refugia during the post-glacial period (11,700 – 8,000 B.P.) (Carrión *et al.*, 2010; Figueiral & Terral, 2002; Terral *et al.*, 2004). The expansion of olive tree populations is thought to have occurred as a result of climate change and was then favoured by human-migratory patterns as suitable habitats became available (Combourieu-

Nebout *et al.*, 2013; Figueiral & Terral, 2002). Palynological records during the Holocene suggest that the abundance of olive trees increased with human activity in the Western and Eastern Mediterranean Basin (Carrión *et al.*, 2010). The exploitation of wild olive trees by humans during the Upper Palaeolithic and Early Neolithic has been reported by several authors (Carrión *et al.*, 2010; Kaniewski *et al.*, 2012; Kislev *et al.*, 1992; Terral, 2000; Terral *et al.*, 2004; Zohary *et al.*, 2012). The early exploitation of olive trees is probably associated with the multiple uses of olives, which include fruit consumption, wood, and cattle fodder (Margaritis, 2013; Renfrew & Cherry, 1972; Terral, 2000). The early exploitation and spread of oleaster populations are thought to represent pre-domestication events, and the practice of pruning oleasters may have largely enhanced flowering and therefore fruit production, an important step towards domestication before agronomic traits were selected for propagation (Margaritis, 2013; Renfrew & Cherry, 1972).

2.4.2 Early domestication of the olive during prehistoric times

Olive tree domestication predominantly refers to the vegetative propagation of the most valuable genotypes, selected for agronomically significant traits (such as greater yield of fruit, larger fruits and higher oil content), and the ease with which they can vegetatively propagated through cuttings or grafting (Besnard *et al.*, 2018). Although live cutting is easy to perform in other species, it is not a feasible method to propagate olive trees as cuttings of cultivars may result in undesired effects; for example, olive cutting of some cultivars may result in high sensitivity to soil pathogens (Besnard *et al.*, 2018). Vegetative propagation by grafting is believed to have favoured the dissemination of olive crops to the Middle East and Central Asia to Western Europe (Juniper & Mabberley, 2006). Breeding of rootstocks in olives is poorly studied compared to apples or grapes (Cornille *et al.*, 2014; Myles *et al.*, 2011; Warschefsky *et al.*, 2016). Previously, combinations of rootstock genotypes were not randomly distributed suggesting some combinations in the Levant area were selected by growers, including enhanced oil quality and drought tolerance (Barazani *et al.*, 2017). During the Quaternary period, the survival of many species was dependent on the existence of favourable conditions or refugia, and patterns of genetic diversity between existing populations are frequently a result of survival in various refugial areas coupled with genetic drift and founder effects during re-colonization (Besnard *et al.*, 2002). Thus, the genetic structure of the olive is most probably the result of numerous factors including the existence of refugia areas, the biogeographic conditions of the Mediterranean Basin, and human-mediated dispersal. In order to clearly understand the domestication of olives, it is essential

to elucidate the origins and the dispersal of the Mediterranean wild olive tree. The domestication of olive trees is proposed to have occurred during the Chalcolithic period (5,700 – 5,500 years ago) in the Near-East and Iberian Peninsula. However, the use of oleasters during the Neolithic period (10,000 – 7,000 years ago) in Spain has been reported (Terral & Arnold-Simard, 1996; Zohary, 1994). The use of oleasters during the Neolithic period is further supported by archaeological studies, which reported this occurrence in several parts of the Mediterranean basin (Liphschitz *et al.*, 1991; Zohary *et al.*, 2012). As olives were used for food, oil, wood and cattle fodder, cultivars could have disseminated towards the Mediterranean basin from the Near-East along with human migrations. Up to the present day, humans have influenced the dispersal of olives by the introduction of cultivars into new areas such as Australia, California, Chile and South Africa (Besnard et al 2007). Oleasters are presumed to be indigenous to the Mediterranean basin (Zohary and Spiegel-Roy 1975) up to southern France (Solari & Vernet, 1992). Presently, it is hypothesized that individual oleaster trees exhibiting superior performance for size and/or oil content of olive fruit were selected, and these selected individuals were vegetatively propagated by planting cuttings directly or grafting cuttings onto indigenous oleasters, producing olive cultivars (Lumaret et al 2004). In the Mediterranean basin, oleaster olives and cultivated clones differ from each other in that oleaster olives possess spinescent juvenile shoots, smaller fruits recognized by less fleshy mesocarp and reduced oil content, including long juvenile stages that may persist for decades in some individuals (Lumaret et al 2004). It has been proposed that hybridization between indigenous oleaster and cultivars introduced from the Levant occurred in the western part of the Mediterranean and could have led to new cultivars (Besnard et al 2001). Furthermore, cultivars may have resulted from some Mediterranean wild populations, but it is also hypothesized that some oleasters resulted from feral forms i.e. individuals that escaped cultivation or resulted from hybridization with cultivars (Besnard *et al.*, 2001; Lumaret *et al.*, 2004).

The genetic history of olives has been difficult to unravel for three reasons: (1) the life-history traits of olive trees is especially challenging compared to annual plants, as olives have a long juvenile phase of 15 to 20 years and adults are able to survive for hundreds of years (Arnan *et al.*, 2012; Fontanazza & Baldoni, 1990) resulting in a slow breeding process that prevents vegetative propagation that allows superior individuals to be maintained and spread (McKey *et al.*, 2010; Miller & Gross, 2011; Zohary & Spiegel-Roy, 1975); (2) the migration of clones produced by vegetative propagation has led to confusion of olive cultivars, such that 1,200 Mediterranean cultivars are of undetermined pedigree (Lumaret *et*

al., 2004); (3) it is possible that hybridization occurred between olive clones and oleasters that survived the Quaternary in the glacial refugia of southern Europe, allowing wild plants to contribute to the genetic diversity of cultivars (Carrión *et al.*, 2010). As unravelling the history of olives is a complex task, several unanswered questions remain. Firstly, was the domestication of olives the result of a single or several event(s)? Secondly, where did domestication event(s) occur? Thirdly, what are the relationships among domesticated cultivars and wild olives, and what is the importance of cultivars and their historical migrations? Lastly, what were the contributions of gene flow between wild olives and cultivars (Lumaret *et al.*, 2004). In spite of the recent accumulation of paleobotanical, archaeological, historical and molecular data contributing to a reassessment of the biogeography of wild olives and the timeline history of their domestication, the processes are not totally understood.

2.4.3 Archaeological and archaeobotanical records

Archaeological records such as milling stones, decantation basins, storage vessels, frescos and ancient writings suggest the introduction of cultivated olives into Greece, Egypt and West Turkey (Vossen, 2007). Cultivated olives continued to migrate westward to Sicily, Sardinia, Italy, France, Spain, Portugal, Algeria, Tunisia, and Morocco. It is thought that Phoenicians took olives to Spain and north Africa, while the Greeks imported olive trees into Italy. Furthermore, the Iberian Peninsula (Spain and Portugal) and the north coast of Africa grew into major production areas of olive oil that was exported to England, Germany, France and Italy, where it was largely used as lamp fuel (Vossen, 2007). The presence of olive trade in human civilizations was observed in the archaeological record during the Chalcolithic period (Galili *et al.*, 1997; Kaniewski *et al.*, 2012; Newton *et al.*, 2014; Zohary *et al.*, 2012). Olive domestication probably followed a dispersal fashion in the Levant, therefore making it challenging to trace the origins solely on the basis of archaeobotanical records. A previous study suggested the early presence of human civilization in localized regions as oleasters growing near inhabitants were harvested, thus suggesting that the exploitation of olives preceded the domestication of olives by several centuries (Kislev, 1994). An abrupt increase in olive seed (stones) remains at eastern and western Mediterranean archaeological sites associated to the Late Bronze Age (3 500 – 3 000 years ago) has been reported; however, morphotypes frequently found in the Middle East were absent in the western archaeological record until 1,000 to 1,500 years later (Newton *et al.*, 2014), confirming the central role that the East to West migrations played in the composition of the diversity of olives in the Western

Mediterranean Basin. Further evidence was found in ancient tombs including written tablets, olive pits, and wood fragments confirming the origin of the cultivated olive in areas along the eastern Mediterranean coast, which is now known as southern Turkey, Syria, Lebanon, Palestine and Israel (Vossen, 2007). Olive oil production continued to increase during the Middle Ages, especially in Spain, Italy and Greece; however, production decreased in North Africa. An upsurge in production was observed after the 1700s, where large olive orchards were established to provide for the growing demand of populations. In the late 19th and 20th centuries, the development of cheap solvent extraction methods for seed oils and the use of other light sources such as gas and electricity became available, resulting in a decrease of olive oil production (Vossen, 2007). Records indicate that various types of seed oils such as cotton, hemp, sesame, palm nuts, sunflower and hazelnut oil were used to contaminate olive oil to make it more cost competitive. This resulted in the establishment of the IOOC in 1959 due to the extensive fraud in olive oil trade surrounding the sale of olive pomace, mislabelled as extra virgin olive oil (Aparicio *et al.*, 1997; Baeten & Aparicio, 2000; International Olive Oil Council (IOOC), 2003).

2.4.4 Molecular evidence for the origin of cultivated olives

Maternal inheritance of cytoplasmic DNA polymorphism provides a suitable tool for phylogeographic and phylogenetic assessments between uniparental lineages dispersed by seeds (McKinnon *et al.*, 2004; Petit *et al.*, 2005; Schaal *et al.*, 1998). Previous molecular studies utilized cytoplasmic DNA polymorphism to assess the origin of wild and cultivated Mediterranean olives (Baldoni *et al.*, 2002; Besnard *et al.*, 2002; Besnard *et al.*, 2000; Vargas & Kadereit, 2001). The use of both cytoplasmic and nuclear markers, especially maternally and biparentally-inherited markers, can aid in the detection of hybridization between distinct populations and reconstructing ancestral lineage sorting events occurring during species formation (Chat *et al.*, 2004; Comes & Abbott, 2001; Cronn *et al.*, 2003; Cronn & Wendel, 2003; Doyle *et al.*, 2004). The internal transcribed spacer (ITS) of the nuclear ribosomal DNA is one of the most commonly used biparentally-inherited markers in angiosperms, as it shows informative polymorphism, and can be amplified and sequenced from poorly preserved material (Álvarez & Wendel, 2003; Baldwin *et al.*, 1995). At present, each subspecies within the olive complex contains a specific plastid lineage/sub-lineage, with numerous lineages/sub-lineages detected within four subspecies (Besnard *et al.*, 2018) (Figure 2.2).

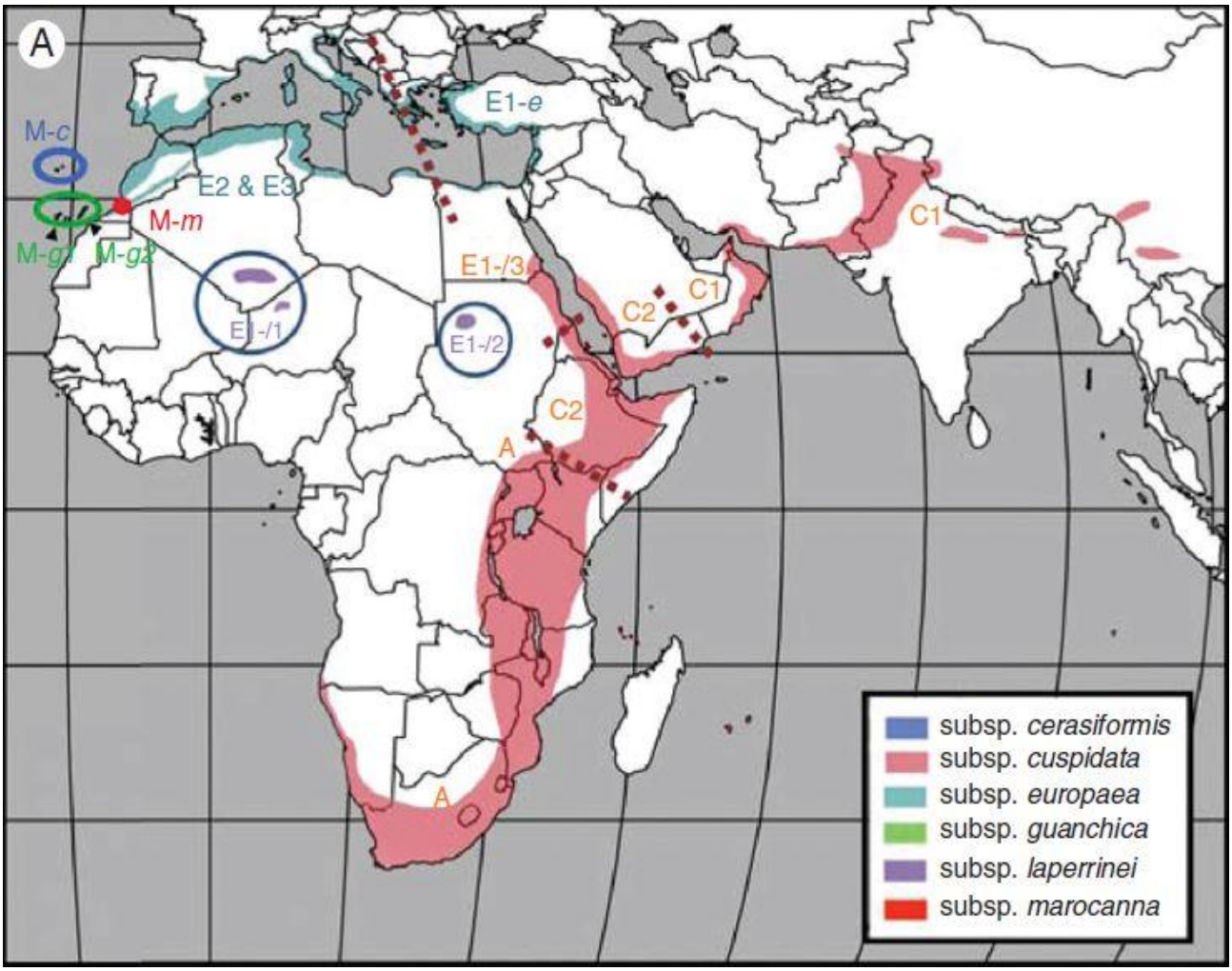

Figure 2.3. Geographical distribution of the six currently recognized subspecies of *Olea europaea* L. (according to Rubio de Casta *et al*. 2006). The dotted lines show estimated limits of the distributions of two adjacent plastid lineages (indicative of presumed secondary contacts). The 13 plastid lineages/sub-lineages currently recognized are specified on the map. Reproduced from Besnard *et al*. 2018.

A previous study comparing chloroplast DNA (cpDNA) variation in the Mediterranean olive and related species found five clades in the cpDNA phylogram (Besnard *et al.*, 2002) as opposed to the three clades observed in previous studies using cpDNA RFLPs (Besnard & Bervillé, 2000; Lumaret *et al.*, 2000). A more recent study using both cytoplasmic and nuclear DNA markers identified two main haplotype lineages (cpI and cpII) and seven sub-lineages. The sub-lineages belonging to cpI comprise of: (1) C1, which includes subsp. *cuspidata* found in eastern Africa and southern Asia; (2) C2, which includes subsp. *cuspidata* found in western Arabia and eastern Africa; (3) A, which includes subsp. *cuspidata* found in tropical and southern Africa. The sub-lineages belonging to cpII comprise of: (4) E1, which include subsp. *europaea, laperrinei* and *cuspidata* found in the Mediterranean and Saharan Africa; (5) E2, which includes subsp. *europaea* found in western Mediterranean; (6) E3, which includes subsp. *europaea* found in western Mediterranean; and lastly (7) M, which include subsp. *guanchica, maroccana* and *cerasiformis* found in

Macaronesia and southern Morocco (Besnard *et al.*, 2007). The distinct geographical distribution of the two main lineages cpI and cpII supports an early differentiation between populations from southern Africa to China (subsp. *cuspidata*) and the Mediterranean, Macaronesia and Sahara (remaining subspecies) (Vargas & Kadereit, 2001). This study was able to recognized three new sub-lineages; a new western Mediterranean lineage (E3) and closely related sub-lineages from southern Asia and eastern Africa (C1 and C2), in addition to the five previously described lineages (Besnard *et al.*, 2002). Furthermore, the sub-lineage C1 is presumed to have diverged from an ancient common ancestor that populated eastern Africa and southern Asia, while the sub-lineage C2 is presumed to have resulted from populations occurring in the African and Arabian coasts of the Red Sea, indicating a secondary divergence from sub-lineage C1 (Besnard *et al.*, 2007). The general distribution of the plastid lineages is incongruent with classical taxonomy (Green, 2002). The majority of the African individuals of subsp. *cuspidata* belonged to sub-lineages A, C1 and C2; however, a population from southern Egypt grouped with sub-lineage E1 (lineage cpII) in eastern Mediterranean (subsp. *cuspidata*) and Saharan (subsp. *laperrinei*). Additionally, subsp. *europaea* populations comprised of three sub-lineages (E1, E2, and E3; lineage cpII), whereas sub-lineage M (lineage cpII) shows a close association of three subspecies (subsp. *guanchica, maroccana* and *cerasiformis*) of north-west Africa and Macaronesia (Besnard, *et al.*, 2007). These results indicate that there are more plastid lineages than documented taxa. Furthermore, a review of the plastid lineages revealed three chlorotypes common to wild olives, (i.e. a haplotype of a chloroplast) associated with sub-lineage E1 (i.e. E1-*e*.1, E1-*e*.2, and E1-*e*.3) depicts 90% of cultivars, which are presently observed in feral olive forms distributed through the Mediterranean Basin. The chlorotypes of the remaining 10% of cultivars are linked to sub-lineages E2 and E3 (Besnard *et al.*, 2018). Moreover, the strong eastern association of the cultivated genepool has been validated using nuclear markers even though a noteworthy contribution of western oleasters have also been shown in the Central and West Mediterranean Basin (Besnard *et al.*, 2001; Besnard *et al.*, 2013; Breton *et al.*, 2006; Diez *et al.*, 2015; Lumaret *et al.*, 2004). Additionally, studies investigating nuclear genetic diversity of cultivated olive indicated a weak genetic structure in the Mediterranean Basin, which is mainly accounted by geographic origin and various uses of cultivars (Belaj *et al.*, 2011; Besnard *et al.*, 2001; Marra *et al.*, 2013). The findings of the molecular studies support the theory of multiple geographic origins of cultivars, but also suggest diversification in the Central and West Mediterranean Basin (Besnard *et al.*i, 2013; Besnard, *et al.*, 2001; Diez *et al.*, 2015).

2.4.5 Cultivated olive introductions into the several non-Mediterranean countries

2.4.5.1 Australia and Hawaii

Cultivated olives were first introduced into Sydney in 1800 (Spennemann & Allen, 2000) and to Adelaide in 1836 (Guerin *et al.*, 2003). The Australian olive industry declined after the 1900s; however, a growing interest in olive products was observed in 1940s and 1950s (Bass *et al.*, 2006). The establishment of naturalized forms reported to have invaded Adelaide (South Australia) occurred before the 1920s. Presently, naturalized populations are observed in other parts of Australia including Western Australia, Victoria, New South Wales and southeast Queensland (Bass *et al.*, 2006) and North New Zealand (Heenan *et al.*, 1999). Furthermore, during the 1990s, the Australian olive industry was revived, which lead to a significant increase in the olive yield. Currently, more than 100 olive cultivars exist in Australia (Sweeney & Davies, 1998). Additionally, *Olea europaea* subsp. *cuspidata* (African wild olive) was introduced into Australia as an ornamental hedging plant (Muyt, 2001), and as rootstock for the European olive. Naturalized populations of the African wild olive were documented in Norfolk Island and regions of Sydney from the 19th century (Spennemann & Allen, 2000). The African wild olive was declared as a persistent environmental weed in eastern Australia that is able to grow in temperate and sub-tropical regions. In Australia, areas including Western Sydney (New South Wales), Illawarra region (NSW), Hunter region (NSW), north-western slopes (NSW), North coast (NSW), Norfolk Island, and Adelaide hills (South Australia) are heavily infested by the African olive (Cuneo & Leishman, 2006). The African wild olive has also invaded and naturalized in the islands of Hawaii, Maui, and Kauai, where it was planted as a windbreaker, and infestation expanded rapidly after the removal of cattle in the early 1970s (Santos *et al.*, 1992). Furthermore, it is also found in New Zealand, presenting the aggressive weed characteristics observed in Norfolk Island. A previous study revealed four plastid haplotypes belonging to both subsp. *europaea* and *cuspidata* were identified in invasive populations from Australia and Hawaii in which populations from South Australia show haplotypes of subsp. *europaea* (Mediterranean taxon), whereas populations from Maui and Campbelltown showed haplotypes of subsp. *cuspidata* (South African populations) (Besnard, Henry, *et al.*, 2007). This data confirms that invasive olive populations in South Australia are in close association with Mediterranean cultivars and populations from Hawaii and NSW are in close association with South African populations, respectively.

2.4.5.2 California

The cultivated olive was first introduced into California by Spanish missionary priests who brought selected cuttings of a particular variety, now known as the Mission variety. In 1870, numerous small orchards with various cultivars were planted for the production of olive oil along the Californian coast from San Diego to Sonoma. The first commercially produced olive oil in California was established in 1871. Furthermore, the “Californian-style” olive, a firm black olive, was developed in northern Sacramento Valley in the early 1990s (Vossen, 2007). During past few years, many olive oil cultivars have been introduced into California (Barranco *et al.*, 2000; Griggs *et al.*, 1975). Furthermore, in 1998, the olive fruit fly was detected in Los Angeles southern California, where it rapidly expanded to the rest of southern California in 1999, the Central Valley in 2000, to Marin, Napa, Sonoma and Solano in 2001, and to Shasta and El Dorado in 2002 (Rice *et al.*, 2003; Collier & van Steenwyk, 2002; Varela & Vossen, 2003).

2.4.5.3 South Africa

Ferdinando Costa, an Italian nurseryman, established an olive industry in Plumstead, Western Cape province of South Africa in 1903 (Costa, 1998). At present, the South African olive industry is over 80 years old with more than 50 commercial olive growers in the Western Cape (Mkize, 2008). SA Olive, formerly known as the SAOGA was initiated in 1956, with the fundamental goal to enrich the South African olive industry. Despite the increase in the South African olive industry, South Africa remains a minor player in the worldwide production of table olives and olive oil production.

2.5 Importance of olive oil and table olives

2.5.1 Processing of olives

The olive tree has been grown since prehistoric times for its fruit that are rich in oils. Previously, olive oil was predominantly used as lamp fuel, but since the late 19th and 20th centuries, the demand for olive oil diminished as affordable solvent extraction methods for seed oils and the use of gas and electricity became available (Uylaşer & Yildiz, 2014). Presently, olive fruit and olive oil offer important nutrients for humans and play an essential role in the economy and culture. The cultivated olive fruit is a drupe, single-seeded indehiscent fruit with a fleshly outer layer. Unripe fruit presents as pale green and changes colour to purple or black when it ripens. Some cultivars are green when ripe whilst others

are copper-brown (Uylaşer & Yildiz, 2014). Table olives are generally harvested in mid-autumn when they are firm and changing colour from green to yellowish green. Contrastingly, the harvest of olives for oil production usually occurs during late-autumn or winter once they have turned black, indicating a decrease in chlorophyll content and an increase in anthocyanin, and when the fruits have reached their maximum oil content (Fedeli & Cortesi, 1993; Haralampidis *et al.*, 1998). Cultivated olives contain oleuropein, an alkaloid responsible for the bitter component, and a low sugar content (2.6% - 6%) compared to other drupes such as peaches, plums and cherries (12% or more). Additionally, olives generally have a high oil content, depending on the variety and time harvested. The olive fruit cannot be consumed directly from the tree due to the oleuropein and requires processing. The most commonly used processing methods include the Californian processing method (Marsilio *et al.*, 2001); the Spanish processing method (Romero *et al.*, 2002; Uylaşer *et al.*, 2008); naturally fermented olives in brine; the Gemlik or Greek processing method (Piga *et al.*, 2001), and naturally fermented olives in dry salt.

2.5.2 Olives in the diet and health

Table olives and olive oil form an essential part of the Mediterranean diet as well as grains, fresh fruit and vegetables, and wine in moderate amounts (Erbay & Icier, 2010; Uylaşer & Yildiz, 2014). As a result, the Mediterranean diet is rich in vitamins, flavonoids and phenolic compounds. Several studies have reported on the low incidence of cardiovascular disease in the Mediterranean region, which is mainly attributed to the consumption of olive products (Keys, 1995; Uylaşer & Yildiz, 2014). The nutritional properties of olives could be attributed to the high content of phenolic compounds, which mainly act as antioxidants and free radical scavengers. The main phenolic compounds present in table olives include tyrosol, hydroxytyrosol, oleanolic acid, and oleuropein. The quality and quantity of phenolic compounds present in table olives is dependent on the processing method, degree of maturation, and the cultivar and irrigation practices.

2.5.3 Phenolic compounds and antioxidant activity

Several studies have been performed to evaluate the phenolic compounds present in table olives. One such study demonstrated the total phenolics level in olive was slightly reduced during the maturation process; however, these compounds were constantly being produced until maturity (Menz & Vriesekoop, 2010). Another study determined the phenolic profile of table olives from a Greek market and concluded, based on their findings of 13 various

polyphenols, that 5-10 table olives may offer the daily recommended intake of polyphenols (Boskou *et al.*, 2006). Phenolic compounds are the most plentiful natural antioxidants in human diet and serve as natural defensive agents against numerous diseases by protecting tissues of the body against oxidative stress. The antioxidant nature of phenolic compounds has been linked to a lower risk of coronary heart disease, lower risk of developing certain cancers, and lower inflammation. The antioxidant activity is predominantly due to the redox properties of phenolic compounds, allowing them to serve as reducing agents, hydrogen donors, and single oxygen quenchers (Ben *et al.*, 2008). Black olives contain a higher level of phenolic compounds therefore presenting higher antioxidant levels (Boskou *et al.*, 2006).

In addition to olive oil serving as a staple food for countries in the Mediterranean region, it has become a favoured source of fat for some health-conscious people who have adopted the Mediterranean diet (Boskou, 2009). Olive oil is rich in oleic acid and is ranked into six categories: extra virgin olive oil, which contains the highest amount of antioxidants; virgin olive oil, which is considered natural as no alterations occur and olives do not undergo any processing; refined olive oil, which is obtained through refining methods and forms the composition of other commercial olive oils; ordinary virgin olive oil, which contains various alterations; olive oil, which is used at the production and wholesale stages; and virgin olive oil not suitable for consumption, also known as lampante virgin olive oil, requires immense processing due to the flawed flavour or aroma (Lastra *et al.*, 2005). Several authors have reported that diets high in oleic acid are as effective in reducing serum cholesterol as diets rich in linoleic acid (Kris-etherton *et al.*, 1999; Mangiapane *et al.*, 1999). Furthermore, an early study examining the lipid metabolism in diabetic rats fed a diet supplemented with olive oil, sunflower oil or fish oil demonstrated that olive oil fed rats showed the lowest levels of triglycerides and highest levels of plasm phospholipids suggesting contributes to an efficient control of the hypertriglyceridemia associated with diabetes compared to the sunflower and fish oil diets (Halliwell *et al.*, 2015).

2.5.4 Medicinal uses and cosmetics

Olives have many medicinal uses, which include relief from muscular spasms, diarrhoea, fever, gout, and headaches. Olive oil have been indicated to be effective in treatment of constipation, diabetes and rheumatism (Uylaşer & Yildiz, 2014). Many studies are centred around the health benefits of olive fruit and olive oil; however, the leaves of olive trees, previously only used as animal feed (Delgado-Pertíñez *et al.*, 2000; Martín-García & Molina-Alcaide, 2008; Martín *etal.*, 2006), have historically used as remedies for fighting fevers and

other diseases such as malaria (Benavente-García *et al.*, 2000), yet olive leaves have not been exploited industrially. A study investigating the phenolic composition of olive leaf extracts reported that the antioxidant capacity of olive leaf extract was greater than vitamin C and E or pure hydroxytyrosol, which a strong antioxidant (Benavente-García *et al.*, 2000). In addition to the health benefits of the phenolic compounds oleuropein and hydroxytyrosol in olive leaves that reduce coronary heart disease and protect against cardiovascular disease (Covas, 2007; Singh *et al.*, 2008; Somova *et al.*, 2004; Visioli & Galli, 1998), oleuropein contains antimicrobial activity against an extensive range of viruses, retroviruses, bacteria, yeast, fungi, moulds and other parasites (Aziz *et al.*, 1998; Juven *et al.*, 1972; Markín *et al.*, 2003). In addition to the health benefits of olives, olive oil has also been reported to have various beneficial effects in cosmetics. Olive oil has been used as a cosmetic and skin protector. Ancient Egyptians used olive oil to make creams and perfumes and it has been proposed that Cleopatra invented the first anti-wrinkle cream using olive oil mixed with milk, incense and juniper berries (Viola & Viola, 2009). Furthermore, the phenolic compounds of olive oil have shown to have a direct antioxidant effect on skin, acting as a free radical scavenger (Ancora *et al.*, 2004). Another essential compound found in high concentration in virgin olive oil is squalene, which has a similar composition as sebum. Squalene serves a powerful scavenger of single oxygen therefore preventing lipoperoxidation caused by ultraviolet rays (Dennis and Shibamoto, 1989; Kohno *et al.*, 1995). Olive oil was reported to offer photoprotection to the skin when taken orally (Cornelli, 2002; Kelly, 1999).

2.6 Olive pests and their control

During the last few decades, the large variation of recent relationships between plants and phytophagous insects has been a subject of evolutionary speculation. An insect is considered phytophagous if its larval or larval and adult stages feed on plant tissues or plant saps. In order to understand the evolutionary aspects of such relationships, it is essential to review the main types: Type A including closely related mono- or oligophagous insect species living on distantly related plant species, which is common in Homoptera, Coleoptera, Lepidoptera, Diptera (*e.g.*Tephritidae), and Hymenoptera (e.g. Chalcidoidea); Type B including closely related oligophagous insect species living on a limited group of plant species belonging to the same plant genera or family, which occurs in Lepidoptera and Coleoptera; Type C including closely related monophagous insect species living on closely related plant species, which is rare (e.g. species of Agaonidae); and type D comprising of

polyphagous insects living on plant species belonging to different families or orders (Jermy, 1984).

The coevolutionary theory as explained by authors (Ehrlich & Raven, 1964) and further developed by many others, is widely accepted and cited as the theory of the evolution of specific insect/host plant relationships, which states: The selection pressure exerted by phytophagous insects on host plants improves the development of plants defense mechanisms. As a result, these plants move into a new adaptive zone and evolutionary radiation of these plants may follow. Furthermore, the phytophagous insects enter a new adaptive zone as well, where they easily diversify without the presence of other rival phytophagous insects. The reciprocal selective responses between insects and plants results in the increase in diversity of both groups of organisms (Jermy, 1984). For example, the evolution of secondary plant substances to phytophagous organisms have been the dominant factors in the evolution of butterflies and other phytophagous groups (Ehrlich & Raven, 1964).

In addition to phytophagous insects and host plant interactions, host-parasitoid interactions are common in natural ecosystems, and parasitoid organisms are regarded as the most important biological control agents used in agriculture and conservation (Henri & Van Veen, 2011). Parasitoid wasps used in pest control of crops, saved the United States agricultural industry an estimated $20 billion per year (Mills & Wajnberg, 2008; Pennacchio & Strand, 2006; Pennisi, 2010). Furthermore, the frequency and importance of host-parasitoid ecological networks *i.e.* the proportion of other species a particular species interacts with, out of all the possible species with which it could interact (Shipley, *et al.*, 2009), have led to vital studies across a range of ecological issues (Ings *et al.*, 2009; Lafferty *et al.*, 2008). Moreover, studies investigating multiple ecological networks is essential as different network types do not exist independently of each other and the structure of one network may have significant effects on the composition of species and the level of interaction in other network types (Ings *et al.*, 2009; Olesen *et al.*, 2010).

2.6.1 Pests associated with olives internationally

2.6.1.1 Olive fruit fly

The olive fruit fly, *Bactrocera oleae* is the major pest of cultivated olives especially in the Mediterranean basin. Regardless of its notoriety on cultivated olives in the Mediterranean, the olive fruit fly is believed to have originated in Africa, where wild olive species exist and

from which domesticated cultivars were derived (Daane & Johnson, 2010a). The distribution of the olive fruit fly is restricted to the areas where cultivated and wild olives are located (Daane & Johnson, 2010). Currently, the olive fruit fly occurs in sub-Saharan Africa, Central America, and Eurasia (Daane & Johnson, 2010; Hoelmer *et al.*, 2011; Mkize *et al.*, 2008). Females lay eggs in both wild and cultivated olives and the newly hatched larvae feed on the pulp of ripening olives, where they either pupate inside the olive or exit to pupate on the ground (Fletcher, 1987; Tzanakakis, 2003). The pest causes significant economic damage for several reasons: (1) oviposition marks on the surface of table olives decrease the quality of the product, (2) fruit drop caused from larvae feeding on the pulp consequently reducing oil production, (3) a diminished quality of pressed oil as a consequence of microorganism growth causing increased acidity, and (4) direct destruction of pulp caused by larvae rendering the fruit unsuitable for consumption as table olives (Daane & Johnson, 2010). Several studies have hypothesized that the evolutionary processes that resulted in the historical distribution and expansion of the olive fruit fly are closely related to the evolution and distribution of the olive tree (Van Asch *et al.*, 2015; Nardi *et al.*, 2005).

2.6.1.2 Olive lace bug

Froggattia olivina (Hemiptera; Tingidae), is a lace bug that has become a serious agricultural pest of cultivated olives in Australia. This species is native to Australia and was first documented feeding on the native olive *Notelaea longifolia* in New South Wales (Froggatt, 1901). In addition, it has been reported to occur in Queensland and Tasmania. Females lay eggs on the underside of leaves in clusters near to the midvein of olive leaves, and excrete a dark-like substance where they feed. The nymphs emerge and cause pinprick holes in the surface of the leaves feeding on the cell contents, which results in yellow spotting on leaves leading to chlorosis (Spooner-Hart *et al.*, 2002).

2.6.2 Pests associated with olives in South Africa

2.6.2.1 Olive fruit fly

The olive fruit fly (Figure 2.4 A) is less detrimental to commercial olive production in South Africa than in the Mediterranean region or California, despite the climate similarity (Hoelmer *et al.*, 2011; Mkize *et al.*, 2008). This may be a result of the action of parasitoid wasps specialized in the olive fruit fly, and present in Southern and Eastern Africa and absent in other world regions. The diversity of parasitoid wasp species was highest in the Western Cape than in any other region in Africa (Copeland *et al.*, 2004; Mkize *et al.*, 2008). Despite

the diversity of parasitoid wasp species in the Western and Eastern Cape, these have been poorly studied (Hoelmer *et al.*, 2011b; Mkize *et al.*, 2008). It has been suggested that the specialized parasitoid wasps found in wild olives in South Africa are responsible for keeping olive fruit fly populations from reaching economic damaging levels in cultivated olives (Caleca *et al.*, 2015). A comparison of larval parasitism in wild and cultivated olives in the Western Cape and the Trapani province (Sicily, Italy) showed a lower level of parasitism in wild olives in the Western Cape compared to Trapani (27% and 48%, respectively). Similarly, cultivated olives in the Western Cape had lower levels of parasitism compared to Trapani (6% and 16%, respectively). The higher parasitism levels in wild olives may be attributed to the fact that parasitoid wasps find it more challenging to reach the olive fruit fly larvae in the dense, thick pulp of cultivated olives (Caleca *et al.*, 2015).

2.6.2.2 Olive seed wasps

In South Africa, olive seed wasps are the main culprits of economic losses in the olive industry, as they result in premature fruit drop. *Eupelmus spermophilus* Silvestri is presently the only confirmed olive seed wasp, and is commonly found in cultivated and wild olives (Figure 2.4 B). Recently, a study identified *E. spermophilus* as the main and only olive seed wasp to infest cultivated olives in the Western Cape province of South Africa (Caleca *et al.*, 2019). Female wasps lay eggs early in season when the inside of the olive seed is soft and allows the larvae to feed on the kernel and pupate. Adult wasps emerge from olives by chewing a hole through the pit and flesh, leaving characteristic emergence holes (Allsopp, 2017).

2.6.2.3 Olive lace bugs

Plerochila australis (Hemiptera: Tingidae) is an olive lace bug, commonly known as olive tingid that has been reported to infest cultivated olives in South Africa. The species is endemic to sub-Saharan Africa where it is distributed from South Africa to northern Ethiopia, probably overlapping with the distribution of wild olive trees (Deckert *et al.*, 2006; Yirgu *et al.*, 2012). Olive lace bugs feed on the underside of the leaves of olive trees causing yellowing of the leaves, leaf fall, and reduce in olive fruit yield. At present, *P. australis* is the only olive lace bug species reported to use wild and cultivated olive trees as its host

2.6.2.4 Olive flea beetles

Presently, three species of olive flea beetles have been known to infest olive trees: *Agropistes oleae* (Bryant), *A. capensis* (Bryant), and *A. sexittatus* (Bryant) (Figures. 2.4 D - 2.4 E). The larvae are leaf miners causing damage to young olive trees and more rarely to the olive fruit (Costa 1998). These species are poorly studied and documented.

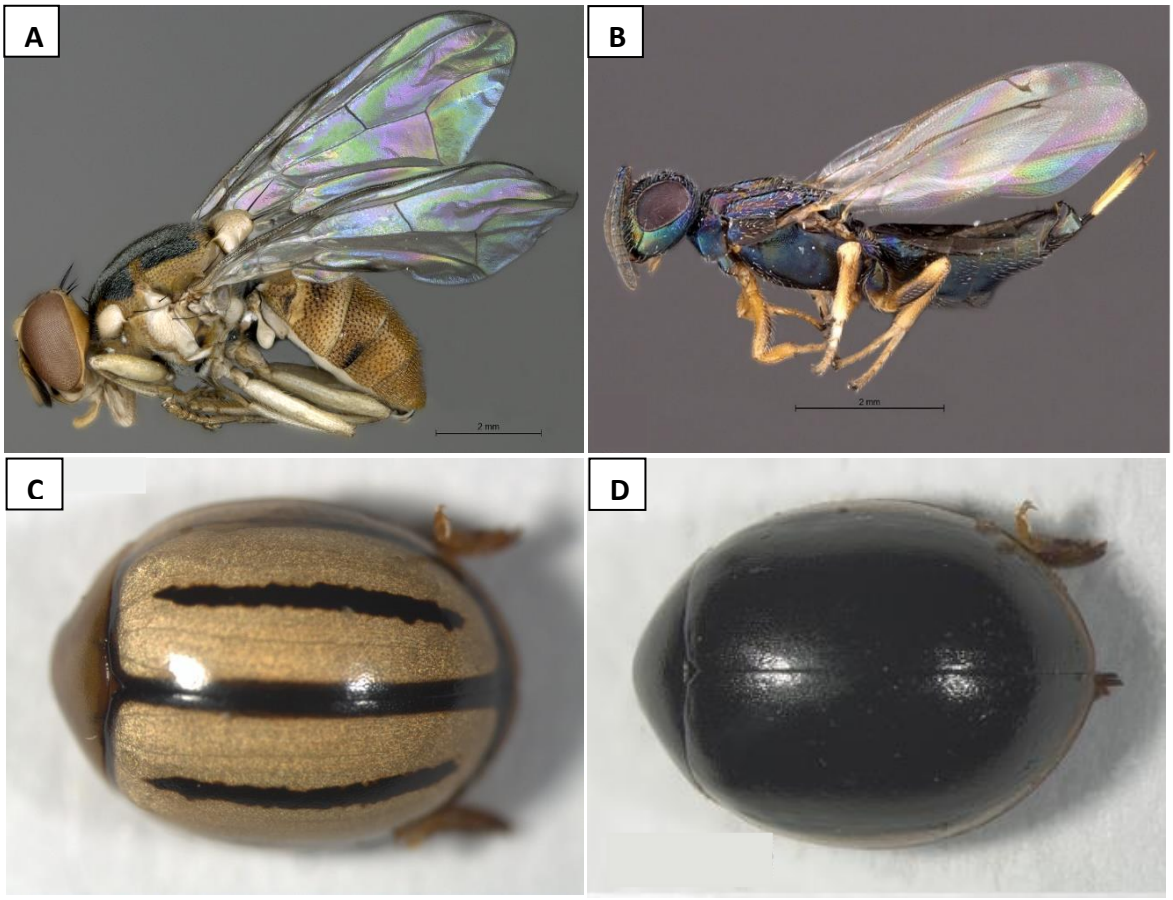

Figure 2.4. Pests associated with olives in South Africa. (A) Olive fruit fly, *Bactrocera oleae*. Imaged by Chanté Powell; (B) Olive seed wasp, *Eupelmus spermophilus*. Imaged by Chanté Powell; Olive flea beetles, *Agropistes oleae* (C) and *Agropistes sexittatus* (D). Imaged by Minette Karsten. No available images for *Plerochila australis* and *Agropistes capensis*.

2.6.3 Olive pest control

The use of insecticides, although very effective for the last 40 years, has been deemed undesirable as residues have been detected in olive oil and olives (Bueno & Jones, 2002). In addition to the detrimental effects of insecticides on the environment, concern has been raised around the effect of insecticides on surrounding beneficial insects (Daane & Johnson, 2010). Therefore, a reduction in the use of conventional insecticides is the goal for many olive-producing regions. Consequently, the focus has shifted to biological control of pest

infestations. As the non-threatening status of the olive fruit fly is presumed to be a result of natural enemies maintaining pest populations at low levels, most surveys searching for olive fruit fly parasitoids were conducted in Africa (Neuenschwander, 1982; Silvestri, 1913, 1914, 1915). Presently, four parasitoid wasp species of the olive fruit fly (*Psyttalia humilis, Psyttalia lounsburyi, Bracon celer* and *Utetes africanus*) are found in South Africa. A renewed interest in the search for parasitoids was initiated after the establishment in of the olive fruit fly California in 2000 (Daane & Johnson, 2010), which led to several studies investigating the performance of *Psyttalia concolor, Psyttalia humilis* and *Psyttalia lounsburyi* (Daane *et al.*, 2015). Due to the limited success of mass-rearing experiments of these species in California, ongoing investigations into parasitoid biology of these species is vital in order to achieve successful biological control applications of the olive fruit fly.

Chapter 3: Barcoding of parasitoid wasps (Braconidae and Chalcidoidea) associated with wild and cultivated olives in the Western Cape of South Africa

3.1 Abstract

Wild and cultivated olives harbor and share a diversity of insects, some of which are considered agricultural pests, such as the olive fruit fly. The assemblage of olive-associated parasitoids and seed wasps is rich and specialized in sub-Saharan Africa, with native species possibly coevolving with their hosts. Although historical entomological surveys reported on the diversity of olive wasp species in the Western Cape Province of South Africa, no comprehensive study has been performed in the region in the molecular era. In this study, a dual approach combining morphological and DNA-based methods was used for the identification of adult specimens reared from olive fruits. Four species of Braconidae and six species of Chalcidoidea were identified, and DNA barcoding methodologies were used to investigate conspecificity among individuals, based on randomly selected representative specimens. Morphological identifications were congruent with DNA data, as NJ and ML trees correctly placed the sequences for each species either at the genus or species level, depending on the available taxa coverage, and low genetic distances strongly supported conspecificity. No clear evidence of cryptic diversity was found. Overall seed infestation and parasitism rates were higher in wild olives compared to cultivated olives, and highest for *Eupelmus spermophilus* and *Utetes africanus*. These results can be used for early DNA-based detection of wasp larvae in olives, and further investigating the biology and ecology of these species.

Keywords: Braconidae, Chalcidoidea, DNA barcoding, olives, species identification

The results of this chapter were published: Powell et al. (2019) Genome *62(3):183-199.*
The supplementary data can be found online at: https://doi.org/10.1139/gen-2018-0068

3.2 Introduction

The wild olive tree (*Olea europaea* L. subsp. *cuspidata*) is closely related to the cultivated olive tree (*Olea europaea* L. subsp. *europaea* var. *europaea*) (Green, 2002). The subspecies is widely distributed on the African continent from the southern tip of Africa to southern Egypt (Rubio de Casas *et al.*, 2006) where it occurs mainly in Afro-montane forests and often near water sources; it is also present in small areas of the Asian continent (Green, 2002). It is known to host a wide variety of leaf-, sap-, and fruit-feeding insects and their associated parasitoids (Copeland *et al.*, 2004; Mkize *et al.*, 2008; Silvestri, 1915). Wild olive trees are often found in close proximity to non-native cultivated olive trees in the Western Cape province of South Africa, the main commercial producing region for olives in the country, due to its typically Mediterranean climate with warm, dry summers and mild, moist winters. The region comprising the Western and the Eastern Cape provinces has been identified as home to a high diversity of wasp species described as natural enemies of olive fruit flies and phytophagous olive seed wasps (Neuenschwander, 1982; Silvestri, 1913, 1915). Two olive fruit flies, *Bactrocera oleae* (Rossi) and *Bactrocera biguttula* (Bezzi), have also been identified and are presently associated with olives in Africa. *Bactrocera oleae*, a major pest of cultivated and wild olives, is believed to have originated and disseminated in Africa, and to have accompanied the geographic expansion and domestication of olive trees in the Mediterranean Basin (Daane & Johnson, 2010; Nardi *et al.*, 2010; Nardi *et al.*, 2005; Zohary, 1994). *Bactrocera biguttula* is a closely related species endemic to the continent, probably also matching the natural range of the geographic distribution of wild olive trees in sub-Saharan Africa (Mkize *et al.*, 2008; Munro, 1926, 1984). Infestation of cultivated olives by *B. biguttula* has never been reported. The infestation rates of cultivated olives by *B. oleae* in South Africa are lower than under similar conditions in the Mediterranean Basin, and the limiting factors have been attributed to the action of indigenous parasitoid wasps (Neuenschwander, 1982; Costa, 1998; Hoelmer *et al.*, 2011) and, more recently, to the specific climatic patterns of the region (Caleca *et al.*, 2015; Caleca *et al.*, 2017; Giacalone, 2011).

The potential utility of parasitoid wasps for the biological control of *B. oleae* in the Mediterranean Basin, where it causes significant damage (Daane & Johnson, 2010), and regions with similar climates such as the Middle East and California, where invasion has occurred more recently (Ramezani *et al.*, 2015; Rice *et al.*, 2003), has sparked

interest in assembling detailed catalogues of southern and eastern African wasp species since the early 20th century (Silvestri, 1915). Surveys conducted in sub-Saharan Africa have reported the presence of a distinct and broad complex of wasps, including species endemic to the region (Hoelmer *et al.*, 2011). Southern European surveys have shown wasp assemblages less diverse and comprised of a smaller number of specialized species. Five parasitoid species are commonly found in southern Europe, of which four are chalcids [(*Eupelmus urozonus* Dalman, *Pnigalio mediterraneus* Ferrière et Delucchi, *Eurytoma martellii* Domenichini, and *Cyrtoptyx latipes* (Rondani), and only one is a braconid [(*Psyttalia concolor* (Szépligeti)]. *Psyttalia concolor* is native to North Africa and some southern Italian regions or sub-regions (Sicily, southern Sardinia, and southern Calabria) (Caleca *et al.*, 2017; Silvestri, 1939) and was purposefully imported into most of southern Europe for the control of *B. oleae* (Borowiec *et al.*, 2012; Hoelmer *et al.*, 2011). Earlier studies provided the first descriptions of wasps associated with wild and cultivated olives in South Africa (the Western Cape, and the Transvaal, a former province that now comprises Gauteng, Limpopo, Mpumalanga, and part of the North-West province) and Eritrea (Neuenschwander, 1982; Silvestri, 1913, 1915). A recent survey in Kenya reported wasps associated with *B. oleae* in wild olives, but only three braconids were identified (Copeland *et al.*, 2004). A more recent study on wild olives in the Eastern Cape province of South Africa reported the occurrence of both parasitoid and seed wasps, and additionally provided estimates of relative infestation rates in wild olives. Four braconids and seven chalcids were found, although some groups were only identified to genus level (Mkize *et al.*, 2008). Similar results were obtained in the Western Cape (Giacalone, 2011; Caleca *et al.*, 2017). None of these works included molecular analyses, and reference DNA barcoding sequences for the majority of the species reported remained unavailable.

The morphological identification of small hymenoptera and requires the expertise of well-trained taxonomists and is difficult to perform on immature life stages. Additional challenges result from sexual dimorphisms, natural intraspecific variation, and the potential presence of cryptic species (Al Khatib *et al.*, 2014; Rowley *et al.*, 2007). DNA barcoding provides a methodological framework for identifying organisms by comparing their degree of nucleotide sequence similarity (expressed as genetic distance) to sets of reference taxa (Hebert, *et al.*, 2003). Sequence similarities can then be interpreted using numerical methods such as hierarchical clustering of genetic distances (e.g., the Neighbour-joining algorithm) and statistical evaluation of thresholds of genetic

distances. The underlying assumption is that interspecific genetic variation exceeds intraspecific variation. DNA barcoding in animals relies on nucleotide sequence similarity at a standard region (~650 bp) of the 5-end of the mitochondrial cytochrome oxidase I gene (*COI*). In recent years, researchers worldwide have been depositing high-quality reference sequences in public databases (e.g., Barcode of Life Data System, BOLD, www. boldsystems.org) (Ratnasingham & Herbet, 2007) that will increasingly allow for the assignment of unknown specimens to morphologically determined taxa, the discrimination of cryptic species, and the elucidation of synonymies (Hebert & Gregory, 2005). Although the potential applications of DNA barcoding are indisputable, methodological limitations and the nature of mitochondrial evolution may restrict its applicability in particular taxa. The use of a single marker also confines the amount of genetic variation, thus limiting the ability to understand the patterns of species boundaries (Dupuis *et al.*, 2012). Another potential limitation of DNA barcoding based on *COI* sequences is the possibility that ancestral polymorphic haplotypes have not sorted according to independent speciation events (incomplete lineage sorting) (Ball *et al.*, 2005). Therefore, it is advisable to combine morphological and DNA-based methods for species identification, as sole reliance on either approach has limitations. The aim of this chapter was to assess the congruence between morphological identification of braconid and chalcid wasps and patterns of genetic clustering and genetic distances within and amongst groups, using novel and publicly available *COI* sequences. The objectives included a sampling strategy that aimed at capturing the total assemblage of wasp species associated with wild and cultivated olives and the assessment of the novel sequences as representative of the species within the context of each particular genus. Additionally, this work also represented an opportunity to report estimates of braconid and chalcid infestation rates across the distribution range of wild and cultivated olive trees in the Western Cape of South Africa, a region known to harbor a rich diversity of these parasitoid and phytophagous wasps.

3.3 Materials and methods

3.3.1 Sample collection

Wild and cultivated olive fruits were collected haphazardly from 16 different areas across the Western Cape province of South Africa and one area in the Eastern Cape province, between March and October 2016 (Figure 3.1). As the objective of this study was to rear, identify, and barcode as many parasitoids and seed wasp species as

possible, and fly and wasp infestation is known to be higher in wild olives, the sampling effort focused particularly on wild olives. Sampling of cultivated olives included un-sprayed fruit collected on commercial farms, as well as in urban areas. Wild olives were collected according to accessibility in diverse contexts, including the vicinity of cultivated olives, wilderness areas, and ornamental trees in urban settings. Olive fruits were stored in ventilated boxes until the emergence of adults. Adult wasps were euthanized by freezing and stored individually in absolute ethanol at −20 °C until DNA extraction. Morphological identification of all specimens was performed on ethanol-preserved adults

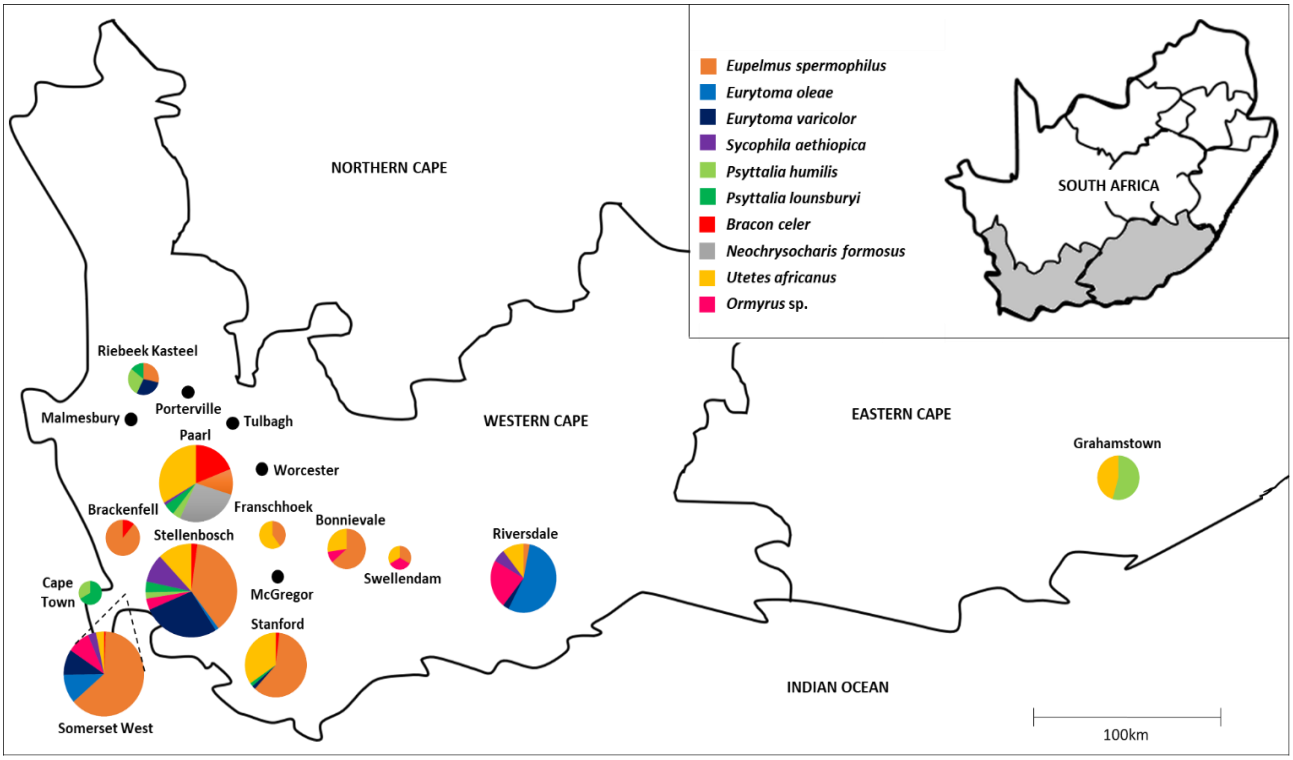

Figure 3.1. Areas of collection of wild and cultivated olives in the Western (16) and Eastern (1) Cape provinces of South Africa. Pie charts represent the relative proportion of Braconidae and Chalcidoidea species reared from olives collected in each area. The size of the circles is proportional to the total number of adult wasp specimens recovered from each area. Black dots represent collection areas from which no specimens were reared.

3.3.2 Morphological identification and photographic imaging

Braconidae were identified to the genus level using the key available in the Parasitoids of Fruit infesting Tephritidae (PAROFFIT) database (http://paroffit.org) (Wharton and Yoder). The genera were identified to the species level following currently available descriptions (Silvestri, 1913) and by comparison to photographic images available on PAROFFIT. Chalcidoidea groups/species were identified as follows: Eurytomidae according to Gates and Delvare (2008) and Lotfalizadeh, Delvare and Rasplus (2007); *Eupelmus* Dahlman according to Al Khatib *et al.* (2014) and Gibson and Fusu (2016),

while the only way to identify specimens at species level for these two families and Eulophidae was to refer to species descriptions provided by Silvestri (1915). Identification of *Ormyrus* Westwood specimens was performed following Boucek, Watsham and Wiebes (1981) and Nieves-Aldrey, Hernández Nieves and Gómez (2007).

Voucher male and female representatives of each species were randomly selected for photographic imaging. Specimens were washed thrice in absolute ethanol with one-hour intervals followed by an additional overnight wash step. Prior to imaging, specimens were processed in a Leica EM CPD300 Critical Point Dryer (Leica Micro-systems, Wetzlar, Germany) to maintain the integrity of morphological structures. Specimens were mounted on felt tips and photographed using a Microscope EntoVision Mobile Imaging System, consisting of a Leica Z16 APO zoom lens attached to a digital camera and computer workstation running on the Leica Application Suite v.4.7.1 (Leica Microsystems). The images were deposited onto BOLD Systems and WaspWeb (www. waspweb.org), an online bioinformatics resource of wasps, bees, and ants documented from the Afrotropical biogeographical region. Female and male specimens were deposited in the entomology collection at the Iziko South African Museum in Cape Town for future reference (Table S1). DNA sequences were not generated from the deposited specimens but from other specimens equally representative of each species, according to morphological identifications.

3.3.3 DNA extraction, PCR amplification, and sequencing

Individual specimens were randomly selected from the total sample of morphologically identified wasps for total DNA extraction and barcoding, and they were subsequently destroyed in the process. A standard phenol- chloroform protocol (Sambrook, Fritsch & Maniatis, 1989) was used for total DNA extraction. The standard *COI* barcoding region (~710 bp) was amplified using the universal invertebrate barcoding primers (LCO1490 and HCO2198) (Folmer *et al.*, 1994) for six species (*Bracon celer*, *Neochrysocharis formosus*, *Psyttalia humilis*, *Psyttalia lounsburyi*, *Sycophila aethiopica*, and *Utetes africanus*). Species-specific primers were designed for *Eupelmus spermophilus* (Eupel-COI-F and Eupel- COI-R), and genus-specific primers were designed for *Eurytoma* (Euryt-COI-F2 and Euryt-COI-R2) (Table S2).

PCR amplifications were performed in 5 µL reactions containing 1× Kapa HiFi

HotStart Ready Mix Kit (KAPA Biosystems), 10 µM of each primer, and 1 µL template DNA. Thermocycling conditions were as follows: initial denaturation at 95°C for 3 min; 5 cycles at 98 °C for 20 s, 41 °C for 15 s, and 72 °C for 1 min, followed by 35 cycles of 98 °C for 20 s, 56 °C for 15 s, 72 °C for 1 min; and a final extension at 72 °C for 10 min. Amplification of the expected fragments was confirmed on a 1.5% agarose gel electrophoresis. PCR products that presented non-specific bands were separated on a 0.8% agarose gel. The correct fragment was then excised from the gel and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) at the Central Analytical Facilities of Stellenbosch University, South Africa. Sequences were manually edited, and homology with known taxa was verified by BLASTn search (www.ncbi.nlm.nih.gov). All sequences were translated into amino acids for the detection of premature stop codons and (or) frameshift mutations indicative of pseudogenes with Geneious R11 (www. geneious.com; Kearse *et al.*, 2012), using the invertebrate mitochondrial genetic code.

3.3.4 Genetic clustering and estimates of sequence divergence

All publicly available *COI* sequences for the Braconidae and Chalcidoidea genera represented in this study were downloaded from GenBank for estimating intra- and interspecific genetic distances and illustrating sequence clustering based on Neighbour-joining (NJ) and Maximum Likelihood (ML) methods (Table S3). Sequences shorter than 500 bp, containing nucleotide ambiguities, and non-overlapping with the *COI* region under study were excluded from downstream analyses. Only sequences identified to the species level were included in the analyses, except in the case of the genus *Sycophila* for which only sequences identified as *Sycophila* sp. were publicly available. To avoid excessively dense trees in the genetic clustering analyses, duplicate haplotypes in the public dataset were identified and deleted using Geneious R11, and a maximum of six sequences were randomly selected when a large number of representatives was available for a single species. For estimates of genetic distances, this last step was not performed (i.e., duplicate sequences were not removed).

Nucleotide sequences were aligned with the MAFFT algorithm implemented in Geneious R11. NJ clustering analyses were performed for each genus in MEGA7 (Kumar *et al.*, 2016) using the Kimura-2-parameter (K2P) model (Kimura, 1980), with

pairwise deletion of the only gap (a 6 bp difference between braconids and chalcids representing two consecutive amino acids). ML trees were reconstructed based on the same alignments with RAxML-HPC Black Box v8.2.10 (Stamatakis, 2014) using the GTRCAT evolutionary model of substitution rate heterogeneity and rapid bootstrapping included in the method (Stamatakis, 2006) and ran on the CIPRES Science Gateway Portal (www.phylo.org; Miller *et al.*, 2010). Clade support in NJ trees was assessed by 1000 bootstrap replications. *Psyttalia humilis* and *B. celer* were used as outgroups for Chalcidoidea, and *E. spermophilus* and *E. varicolor* were used as outgroups for Braconidae. Estimates of intra- and interspecific sequence divergence and relative standard errors were estimated in MEGA7 using the K2P model.

3.3.5 Infestation rates

Apparent parasitism rates (APR) for Braconidae species and *Neochrysocharis formosus* were estimated as follows: APR = total number of adult specimens of the particular species/ (total number of tephritid flies + total number of adult braconid and *N. formosus* specimens). For Chalcidoidea species, apparent seed infestation rates (AIR) were estimated as follows: AIR = total number of adult specimens of the particular species/total number of olives. For the sake of simplicity, apparent parasitism and apparent seed infestation rates will be generically designated as infestation rates (IFs) in this report.

3.4 Results

3.4.1 Sample collection survey

A total of 83 381 olive fruits (wild = 76 960 and cultivated = 6421) were haphazardly collected in 16 areas in the Western Cape province and one area in the Eastern Cape province of South Africa between March and October 2016 (Table S4). No adult wasp specimens were reared from wild or cultivated olive fruits collected in two areas (Tulbagh and Worcester) or from cultivated olives collected in five areas (Malmesbury, McGregor, Porterville, Riebeek Kasteel, and Somerset West). A total of 843 adult wasp specimens was reared from wild (*n* = 836, 99.2%) and cultivated olives (*n* = 7, 0.8%). Based on morphology, specimens were distributed among six species of Chalcidoidea (*Eupelmus spermophilus*, *n* = 321 (38.1%); *Eurytoma oleae*, *n* = 58 (6.9%); *Eurytoma varicolor*, *n* = 136 (16.1%); *Sycophila aethiopica*, *n* = 50 (5.9%); *Neochrysocharis formosus*, *n* = 23 (2.7%); *Ormyrus* sp., *n* = 47 (5.6%)) and four species of Braconidae (*Psyttalia humilis*, *n* = 28 (3.3%);

Psyttalia lounsburyi, *n* = 22 (2.6%); *Utetes africanus*, *n* = 130 (15.4%); *Bracon celer*, *n* = 28 (3.3%)). Photographic images of one male and one female specimen representative of each species are presented in Figures 3.2. – 3.5.

Figure 3.2. (A) *Bracon celer* female; (B) *Bracon celer* male; (C) *Psyttalia humilis* female; (D) *Psyttalia humilis* male.

Figure 3.3. (A) *Psyttalia lounsburyi* female; (B) *Psyttalia lounsburyi* male; (C*) Utetes africanus* female; (D) *Utetes africanus* male.

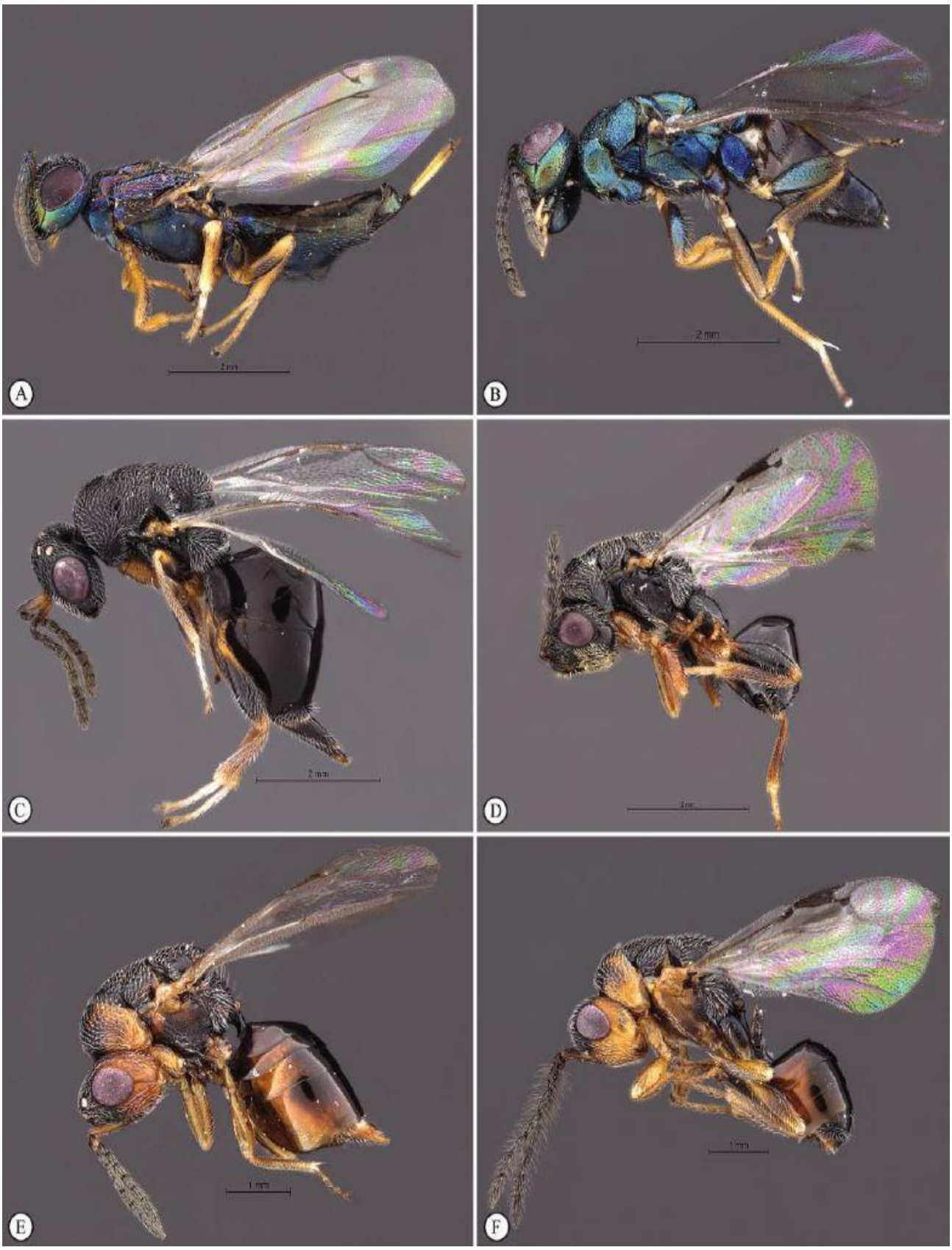

Figure 3.4. (A) *Eupelmus spermophilus* female; (B) *Eupelmus spermophilus* male; (C) *Eurytoma oleae* female; (D) *Eurytoma oleae* male; (E) *Eurytoma varicolor* female; (F) *Eurytoma varicolor* male.

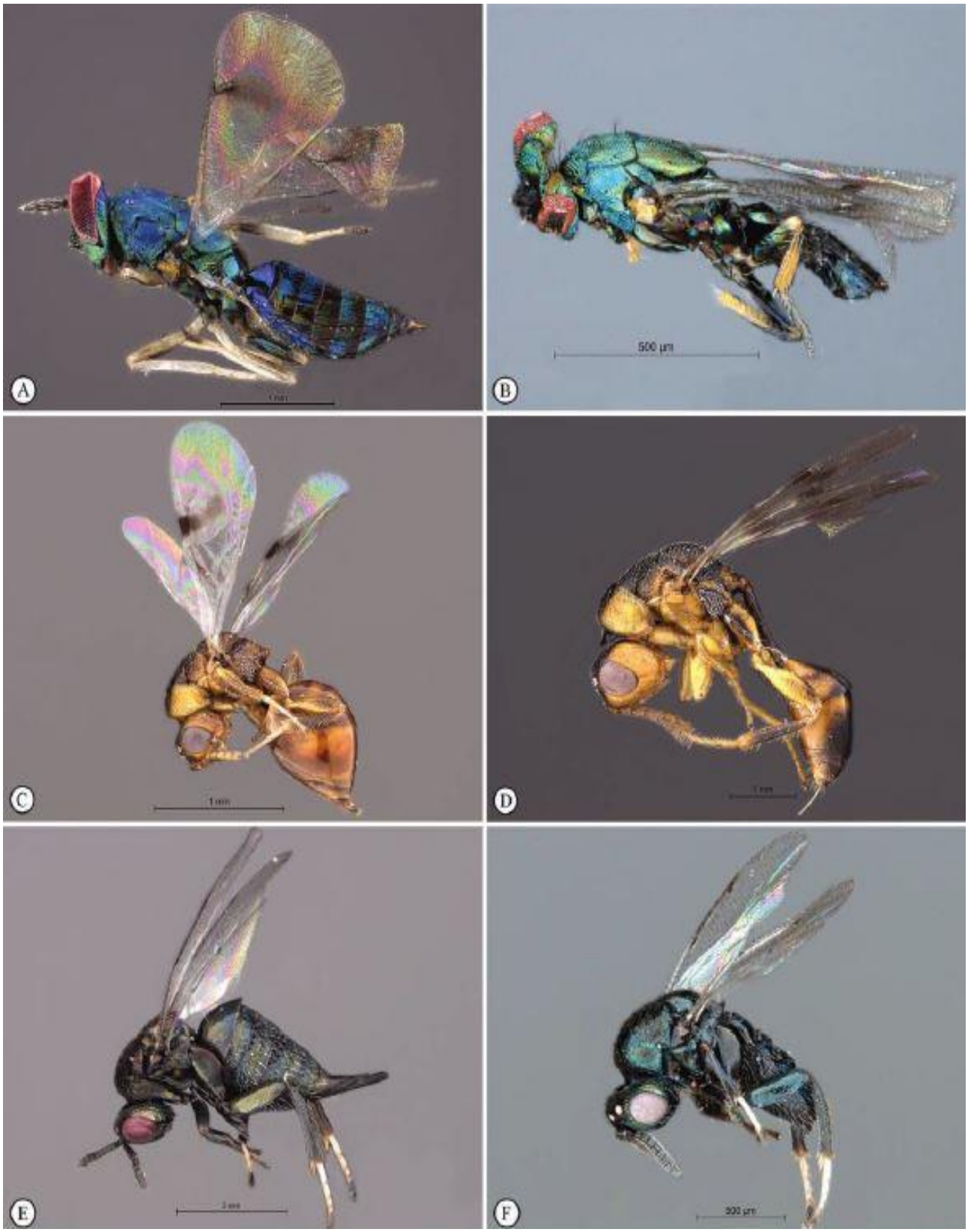

Figure 3.5. (A) *Neochrysocharis formosus* female; (B) *Neochrysocharis formosus* male; (C) *Sycophila aethiopica* female; (D) *Sycophila aethiopica* male; (E) *Ormyrus* sp. female; (F) *Ormyrus* sp. male.

Overall, Chalcidoidea (*n* = 635, 75.3%) were more abundant than Braconidae (*n* = 208, 24.7%). The most abundantly reared chalcid was *E. spermophilus*, and the most abundant braconid was *U. africanus*. Only three species were recovered from a total of 2,583 cultivated olives in three areas (Franschhoek, Paarl, and Stellenbosch): *E. spermophilus* (*n* = 4), *B. celer* (*n* = 2), and *P. lounsburyi* (*n* = 1) (Figure 3.6).

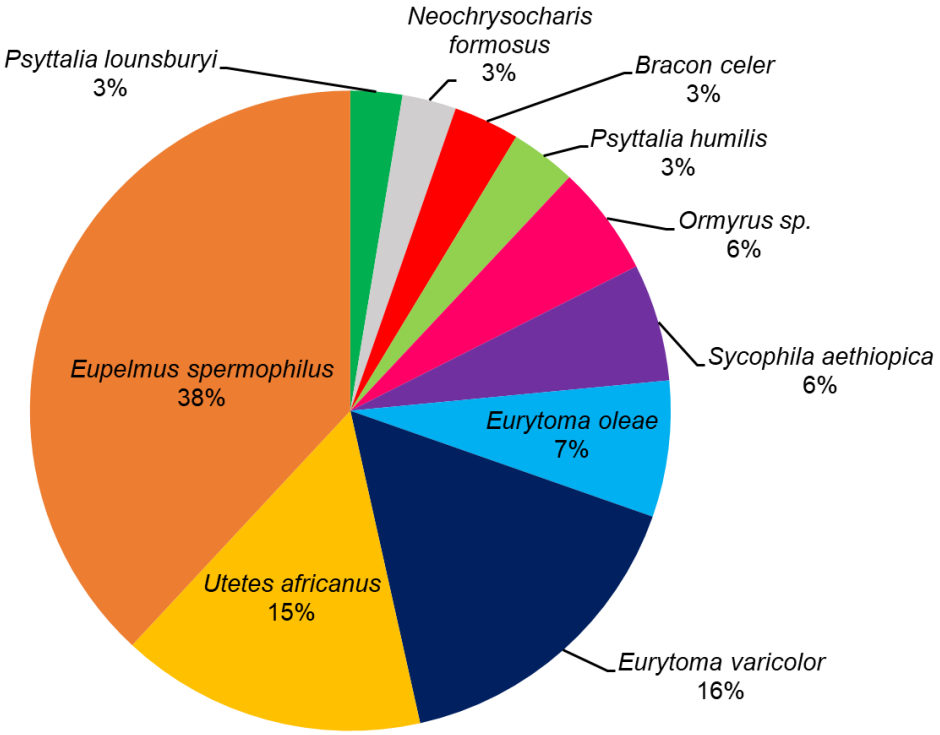

Figure 3.6. Relative proportions of adult braconid and chalcid wasps reared from wild and cultivated olives collected in 16 areas in the Western Cape and one area in the Eastern Cape provinces of South Africa.

3.4.2 PCR amplification and sequencing

PCR amplification using the universal invertebrate barcoding primers LCO1490 and HCO2198 only generated the expected product in DNA samples from *P. humilis*, *P. lounsburyi*, *B. celer*, and *S. aethiopica*. *Eupelmus spermophilus* and *Eurytoma* species did not consistently amplify with these primers; therefore, new primers were designed for the amplification of a shorter amplicon (650 bp) within the standard *COI* barcoding region (Table S2). The new Euryt-COI-F2 and Euryt-COI-R2 primers generated non-specific products in *E. varicolor*; therefore, purification of the specific band from a 0.8% agarose gel was performed prior to sequencing reactions. *Utetes africanus* also presented non-specific amplifications with the universal primers, and purification of the specific band from a 0.8% agarose gel was necessary.

One *Wolbachia* sequence, identified by BLASTn search during the sequence quality control

procedure, was unintentionally obtained from an *E. spermophilus* DNA sample using the universal primers. These primers did not consistently generate PCR products in *E. spermophilus*, and were subsequently replaced by newly designed species-specific primers (Eupel-COI-F and Eupel-COI-R, Table S2). The species-specific primers were then used for generating the DNA data presented in this study, and did not amplify *Wolbachia* sequences. Three putative pseudogene fragments, amplified from *E. varicolor* using the Euryt-COI-F2/Euryt-COI-R2 primer pair, were also detected during the sequence quality control procedure. These sequences were similar to the functional *COI* region, but amino acid translation showed several stop codons; therefore, they were excluded from downstream analyses. Novel reference barcoding sequences were generated for six of the nine species identified in this study: *B. celer* (*n* = 1), *U. africanus* (*n* = 10), *E. spermophilus* (*n* = 10), *E. oleae* (*n* = 9), *E. varicolor* (*n* = 6), and *S. aethiopica* (*n* = 1). Additional sequences were generated for *P. lounsburyi (n* = 4), *P. humilis* (*n* = 3), and *N. formosus* (*n* = 2) (Table S5). All sequences with the corresponding trace files, specimen images, GPS coordinates, and biological data were deposited on BOLD (projects UTET, SYCPH, PSYT, NCHRY, EURYT, EUPEL, and BRCN) and made publicly available. All sequences were also deposited in GenBank (Table S3).

For an overview of the current coverage of Braconidae and Chalcidoidea, two separate family trees were constructed, with posterior condensation of species clusters into single branches (Figures 3.7 and 3.8). Genus-specific trees were also constructed to provide a detailed illustration of the relationships between the individual sequences generated in this study (Figs. S1–S7). Species clusters were strongly supported by the NJ distance-based method and were in agreement with the ML analysis; therefore, only NJ-based trees are shown, with reference to the relevant topological differences in the text.

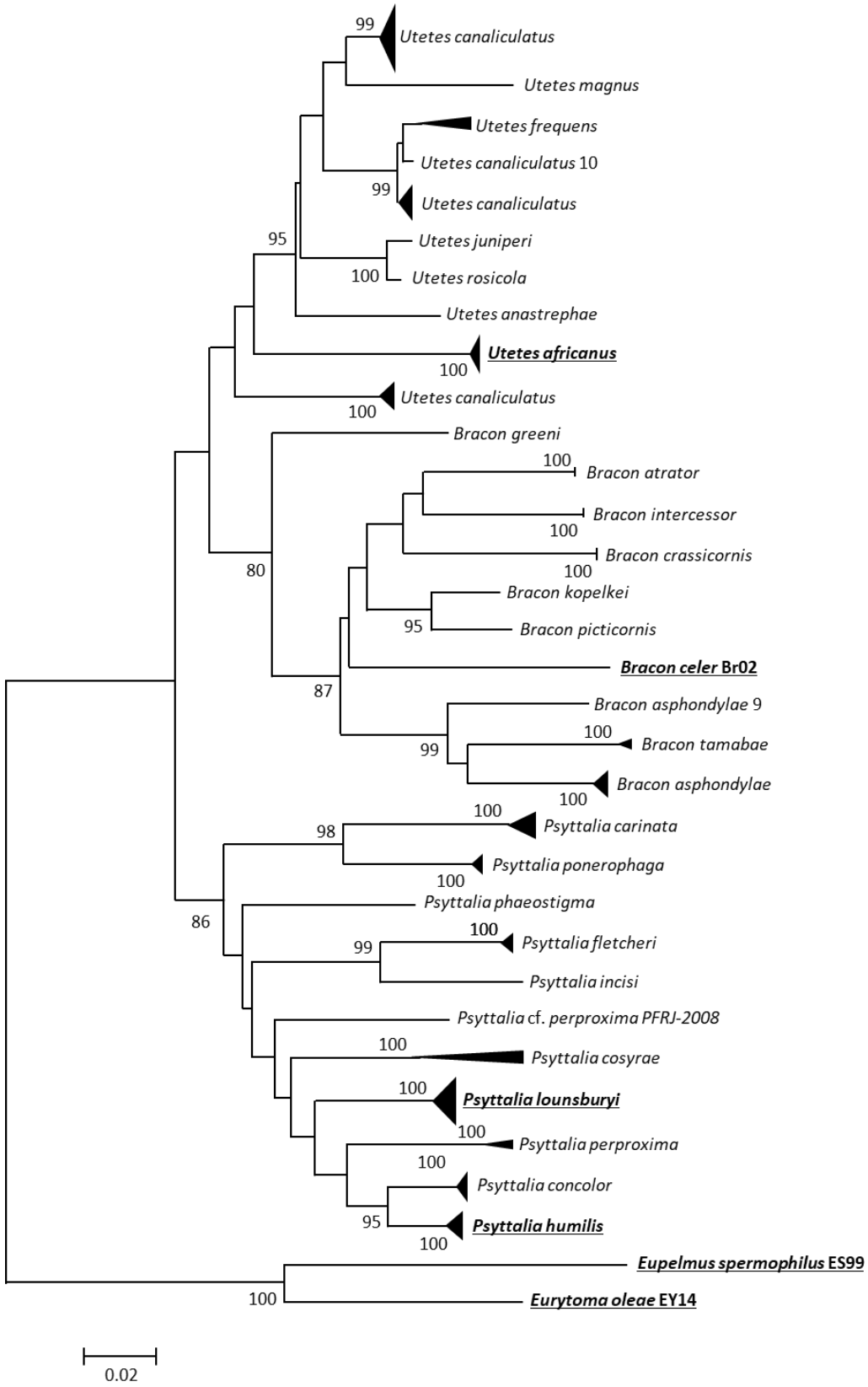

Figure 3.7. Neighbour-joining (K2P) tree of the family Braconidae, based on a 485 bp alignment of 128 COI sequences and two outgroups, with pairwise deletion of sites. Values indicate nodal bootstrap support (1,000 replicates). The scale bar represents the percentage of sequence divergence. Species surveyed in this study are shown in bold and underlined. Triangles represent condensed species clades.

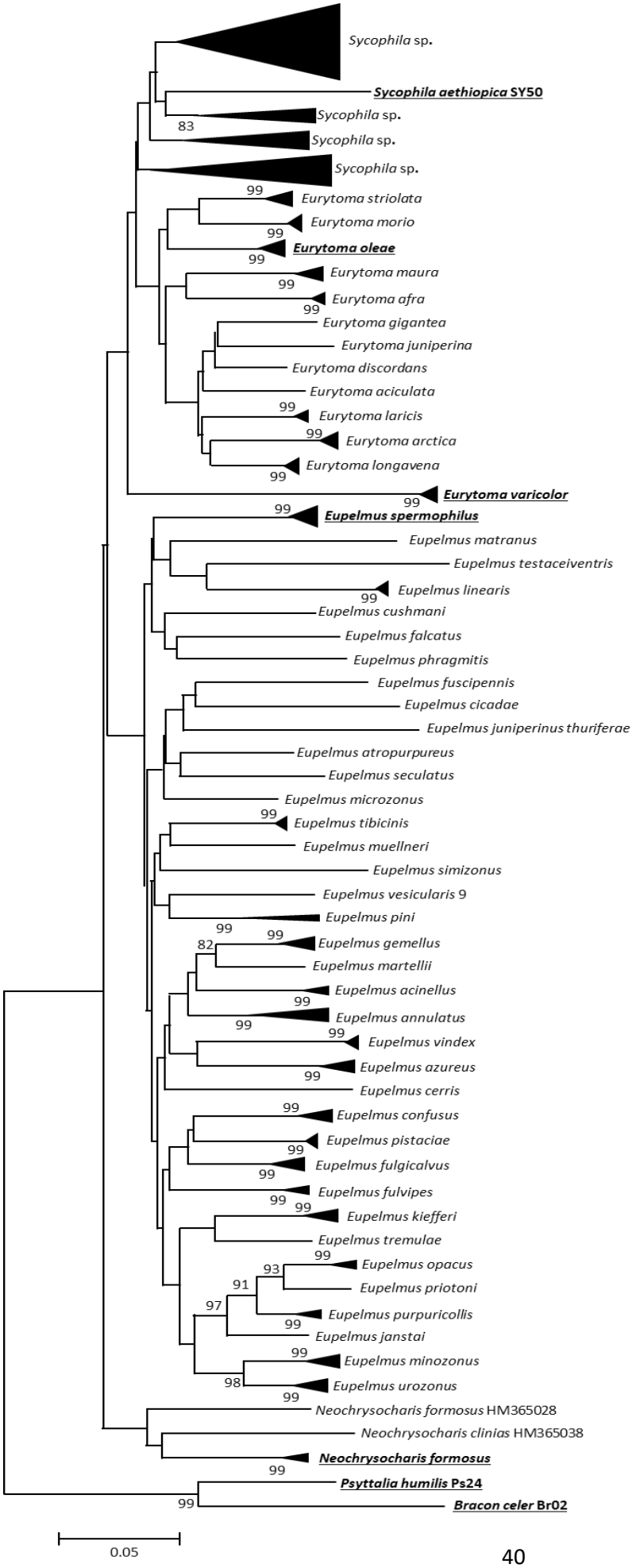

Figure 3.8. Neighbour-joining (K2P) tree of the superfamily Chalcidoidea, based on a 460 bp alignment of 225 COI sequences and two outgroups, with pairwise deletion sites. Values indicate nodal bootstrap support (1,000 replicates). The scale bar represents the percentage of sequence divergence. Species surveyed in this study are shown in bold and underlined. Triangles represent condensed species clades.

3.4.3 Braconidae

Bracon celer Szépligeti (Figures. 3.2 A – 3.2 B) represented 13.5% of the total braconids, similarly to *P. humilis* and *P. lounsburyi*. *Bracon celer* was reared almost exclusively from wild olives, with a single specimen found in cultivated olives from Paarl (Table S4[2]). The genetic clustering analyses for the genus *Bracon* included 22 sequences distributed among nine species, with four species represented by a single sequence. NJ and ML trees recovered identical topology and showed *B. celer* (*n* = 1, this study) nested as an internal branch. The genetic clustering was consistent with species designations, except for the non- monophyly with strong nodal support for *B. asphondyliae* (Figure S1).

Psyttalia humilis (Silvestri) (Figures. 3.2 C – 3.2 D) was reared exclusively from wild olives in five areas, and represented 13.5% of all braconids, whereas *P. lounsburyi* (Figures. 3.3 A – 3.3 B) was reared from both cultivated (a single specimen in Paarl) and wild olives in five areas, and represented 10.6% of all braconids (Table S4). The NJ and ML analyses for the genus *Psyttalia* included 54 sequences distributed among 11 species, with only three species represented by a single sequence. The topology of the trees showed monophyletic clustering of sequences in congruence with species designations (Fig. S2). The *P. humilis* (*n* = 3) and *P. lounsburyi* (*n* = 4) specimens identified and sequenced in this survey grouped with the publicly available sequences in their respective monophyletic clusters. The same pairs of sister species were recovered in NJ and ML (e.g. *P. lounsburyi/P. phaeostigma*; *P. humilis/P. concolor*), although the topology of the deeper branches differed, albeit with low statistical support. Interspecific sequence divergence ranged between 8.3% for the species pair *P. carinata/P. ponephoraga* and 16.3% for *P. carinata/P. fletcheri.* Intraspecific sequence divergence was estimated for six species, and maximum distances were lower than 1.7% in all cases (Table S6). Intraspecific genetic distances were also estimated, separating the new *P. lounsburyi* and *P. humilis* sequences from the conspecific sequences available on GenBank. No differences (i.e. high genetic distances) were found; therefore, the public dataset did not seem to include sequences incorrectly assigned to species.

Utetes africanus (Szépligeti) (Figures. 3.3 C – 3.3 D) was the most abundantly reared braconid (62.5%), and was exclusively found in wild olives in nine areas (Table S4). The NJ and ML trees for the genus *Utetes* included 52 sequences distributed among eight species, with four species represented by a single sequence. *Utetes canaliculatus* (*n* = 34) represented 65.4% of the total sequence dataset for the genus (Figure S3). The NJ and ML trees showed

the same topology, except for the position of *U. magnus*. A polyphyletic pattern was recovered for *U. canaliculatus*, with a highly diverged monophyletic group (cluster 3), a polyphyletic group including *U. frequens* (cluster 2), and a monophyletic (in NJ) or polyphyletic (in ML, where it included *U. magnus*) cluster 1 (Figure S3). The maximum intraspecific genetic divergence considering *U. canaliculatus* as a single group was 11.0%, whereas for each of the separate clusters it was lower than 0.7%. The divergence was highest between cluster 3 and the other two clusters, and the lowest between clusters 1 and 2 (Table S7), as suggested by the topology of the tree. *Utetes africanus* (*n* = 10, this study) formed a monophyletic cluster, and the sequences had high similarity (maximum intraspecific distance = 0.4%).

3.4.4 Chalcidoidea

Eupelmus spermophilus Silvestri (Figures. 3.4. A – 3.4. B) represented 50.6% of the total chalcids, and was recovered from the three areas where wasps were reared from cultivated olives, and in nine areas from wild olives (Table S4). The NJ and ML trees of the genus *Eupelmus* included 99 sequences distributed among 37 species, with 18 species represented by a single sequence (Figure. S4). The general topology of the trees recovered monophyletic clustering for all *Eupelmus* species, including *E. spermophilus* (*n* = 10, this study), albeit with different topology and low statistical support of deeper nodes in ML and NJ. Intraspecific sequence divergence was estimated for nine species (Table S8). Maximum intraspecific distances ranged between 2.3% for *E. spermophilus* and 8.7% for *E. annulatus*. Interspecific sequence divergence ranged between 16.9% for the species pair *E. spermophilus*/*E. azureus* and 7.8% for the lowest pair *E. minozonus*/*E. urozonus*.

Eurytoma oleae Silvestri (Figures. 3.4. C – 3.4. D) represented 9.1% of the total chalcids, and was reared exclusively from wild olives in three areas (Table S4). The highest IF was found in Riversdale (1.89%), whereas the average was 0.10% in the other areas. *Eurytoma varicolor* Silvestri (Figures. 3.4. E – 3.4. F) was reared exclusively from wild olives in five areas, and represented 21.4% of the total chalcids (Table S4). The NJ and ML trees for the genus *Eurytoma* comprised 59 sequences distributed among 16 species, with only four species represented by a single sequence. Although deeper nodes had different topologies with low statistical support in NJ and ML, all species formed monophyletic clusters, including *E. oleae* and *E. varicolor* (*n* = 9 and *n* = 6, respectively, this study) (Figure. S5). All maximum intraspecific distances were lower than 2.7%, and interspecific distances were higher than 7.7% (*E. morio*/*E. striolata*), with the

highest between *E. oleae* and *E. varicolor* (18.1%) (Table S9).

Sycophila aethiopica (Silvestri) (Figures. 3.5. C –3.5. D) was reared exclusively from wild olives in four areas, and represented 7.9% of the total chalcids (Table S4). The NJ and ML trees of the genus *Sycophila* included 71 sequences, with only one sequence identified to the species level (*S. aethiopica*, this study). Identical sequence clusters were recovered in NJ and ML, albeit with different topology and low statistical support of deeper nodes. *Sycophila aethiopica* nested in the interior branches of the trees (Figure. S6).

Neochrysocharis formosus (Westwood) (Figures. 3.5. A – 3.5. B) was reared exclusively from wild olives in Paarl (*n* = 23), and represented the lowest proportion (3.6%) of the total chalcids (Table S4). A previous phylogeny of Eulophidae based on morphological and molecular markers (*COI* and 28S rRNA D2-D5) showed that *N. formosus* and *N. clinias* were a paraphyletic group with respect to *Asecodes* sp., although the study included a single sequence for each *Neochrysocharis* species from Italy (Burks *et al.*, 2011). Due to the poor sequence coverage of the genus *Neochrysocharis*, public *Asecodes* sequences with high quality (*A. lucens*, *n* = 6) were included in the NJ and ML analyses, along with the available sequences for *Neochrysocharis* identified to the species level (*N. formosus*, *n* = 3; and *N. clinias*, *n* = 1). The NJ and ML trees recovered *N. formosus* (*n* = 2, this study) and *A. lucens* as sister species with high statistical support. However, *N. formosus* HM365028 (GenBank) did not cluster with the new *N. formosus* sequences (Figure. S7). Genetic distance estimates showed that the maximum divergence between the three *N. formosus* sequences was 12.9% (Table S10). A closer inspection revealed that *N. formosus* HM365028 was highly polymorphic relative to the two new *N. formosus* sequences, which diverged between them by only 1.1%.

Ormyrus sp. (Figures. 3.5. E – 3.5. F) was reared exclusively from wild olives in five areas, and represented 7.4% of the total chalcids (Table S4). Identification to the genus level (*Ormyrus* Westwood) was performed using solely morphological characters, as molecular analyses were not successful. Although PCR amplification products were generated and sequenced, BLASTn search resulted in no matches with known *COI* sequences, or any other sequence. Overall, apparent parasitism rate was the highest for *U. africanus* (11.80%) (Figure. S8). *Psyttalia*, *B. celer*, and *N. formosus* had approximately five-fold lower IFs (2.54% for *P. humilis*, 2.00% for *P. lounsburyi*, 2.54% for *B. celer*, and 2.09% for *N. formosus*). Apparent seed infestation rate was the highest for *E.*

spermophilus (0.38%). *Eurytoma oleae*, *S. aethiopica*, and *Ormyrus* sp. had similar IFs (average 0.06%), and *E. varicolor* had an intermediate IF of 0.16%. A richer wasp assemblage was reared from wild olives compared to cultivated olives, from which only two braconids (*B. celer* and *P. lounsburyi*) and one chalcid (*E. spermophilus*) were reared at low IFs in three areas (Franschhoek, Paarl, and Stellenbosch) (Figures. S9 –S10).

3.5 Discussion

3.5.1 Sample collection survey

In this study, 10 wasp species (four braconids and six chalcids) were reared from wild and cultivated olives and identified based on morphological characters following the currently available keys. All groups reported in a previous survey of wild olives performed in the Eastern Cape (Mkize *et al.*, 2008) were observed, except for *N. formosus*, which was only recovered in the present survey. Resolution to the species level was improved for two of the genera reported in the Eastern Cape: *Eurytoma*, with the identification of *E. oleae* and *E. varicolor*, and *Sycophila*, with the identification of *S. aethiopica*. *Eupelmus afer* was reportedly reared in the Eastern Cape (Mkize *et al.*, 2008), but it was not identified among the specimens reared in the present study.

The sampling strategy was haphazard and opportunistic, and not designed to consistently survey and compare the assemblage of species in wild and cultivated olives in the Western Cape, but to potentiate the rearing of the widest possible range of wasps for morphological species identification and DNA barcoding. Some areas were visited only once, while other areas were sampled multiple times (e.g., Paarl and Stellenbosch). Additionally, the number of cultivated olives collected was much lower than the number of wild olives, for which more areas were also sampled. This sampling bias was deliberate because wild olives are known to harbor more olive flies, braconids, and chalcids than cultivated olives. The low presence of braconids in cultivated olives is most probably due to three main reasons. First, olive fruit fly infestation is relatively low in the Western Cape, thus precluding high levels of parasitoid wasp populations. Second, braconids have difficulty reaching the third instar fly larvae, the larval stage most attacked by parasitoids, as third instar larvae feed close to the olive kernel, and the thick pulp layer of cultivated olives limits the action of parasitoids. This is especially relevant in the case of *U. africanus*, a species that has a very short ovipositor. As for chalcids, seed wasps attack olives when the fruit is small and the kernel is still soft, but

infestation is also limited because of the thicker pulp layer of cultivated olives compared to wild olives. The atypical climatic conditions of extremely low rainfall in the Western and the Eastern Cape provinces since 2015 may have also contributed to the absence of wasps in olives collected in five areas, and to non-representative infestation rates. Therefore, the estimates for apparent parasitism and seed infestation rates here presented should be interpreted with caution. However, it is relevant to note that *U. africanus* was the most abundantly reared braconid, and *E. spermophilus* was the most abundant chalcid, with the latter the most abundant wasp overall (38%). It is also relevant to note that, despite the haphazard olive sampling across the Western Cape, a higher diversity of species and higher IFs were found in wild olives than in cultivated olives, as expected.

3.5.2 Morphological identification vs DNA-based identification

At least one specimen per species was sequenced for the standard barcoding *COI* region for all species, except for *Ormyrus* sp. These nucleotide sequences represent the first DNA barcoding references for all species except *N. formosus* and the two *Psyttalia* species, for which at least one sequence was publicly available. The consistency between sequence similarity and morphological identification was then investigated using K2P distances and NJ and ML trees. Phylogenetic reconstruction and estimates of genetic distances offer useful insights into evolutionary relationships among taxa, thus assisting species identification, provided that the specific taxonomic group is well represented in the reference dataset (Collins *et al.*, 2012; Hebert *et al.*, 2003; Hebert *et al.*, 2004; Ross *et al.*, 2003; Ross *et al.*, 2008). This was not the case for all species identified in this study. For example, publicly available sequences for the genus *Sycophila*, although abundant (*n* = 70), were not identified to the species level. The genus *Bracon* followed a pattern of incomplete identification: 75% of the 447 public *COI* sequences were identified as *Bracon* sp., and 68% of the sequences identified to the species level were duplicates (i.e., sequences with identical residues), resulting in a final dataset of 22 overlapping *COI* sequences. *Neochrysocharis* was similarly covered, as 34% of the public sequences were only identified to the genus level.

Additionally, after the removal of duplicates, 92% of the remaining 24 *Neochrysocharis* public sequences identified to the species level had a short overlap with the standard *COI* barcoding region. Therefore, the final dataset for the genus *Neochrysocharis* included only the four sequences used in the NJ and ML analyses. These difficulties

highlight the importance of good taxonomic coverage for the generation of reliable species reference sequences. In the context of the purpose of this study, which aimed at associating morphologically identified specimens with DNA barcodes, phylogenetic reconstruction using the ML methodology did not show improved resolution or reliability over the distance-based NJ method, as NJ and ML recovered the same monophyletic species clusters with high statistical support. Deeper nodes, on the other hand, were as poorly supported in NJ as in ML, as expected when using relatively short sequences (~500 bp) of closely related species (Min & Hickey, 2007).

Overall, we found complete concordance between morphological identification of specimens, sequence clusters on NJ and ML trees, and genetic distances for six species (*E. spermophilus*, *E. oleae*, *E. varicolor*, *P. humilis*, *P. lounsburyi*, and *U. africanus*) of the 10 species reared from olives. No clear evidence for cryptic diversity was found, as these species formed monophyletic clusters with high bootstrap support, and maximum intraspecific genetic distances were within the range of the commonly used barcoding thresholds of 2%–3%, and lower than 1.3% in all cases, except for *E. oleae* (2.7%). Interestingly, the maximum intraspecific genetic distance in *E. spermophilus* was 2.3%, the lowest in the genus *Eupelmus*, for which high intraspecific divergence was found, with the most striking case being *E. annulatus* (8.7%).

3.5.3 Braconidae wasp species

Bracon celer is an idiobiont ectoparasitoid of third (last) instar olive fruit fly larva, and the only *Bracon* species known to be an olive fruit fly parasitoid (Silvestri, 1913). In sub-Saharan Africa, *B. celer* has been reported in Kenya, Ethiopia, Namibia, and South Africa (Daane *et al.*, 2011; Mkize *et al.*, 2008; Neuenschwander, 1982; Silvestri, 1913, 1915). The genus was previously found to be monophyletic with high statistical support, using 658 bp *COI* sequences (Matsuo *et al.*, 2016). Our NJ and ML analyses recovered

B. asphondilae and *B. tamabae* as non-monophyletic with low statistical support, probably due to the shorter *COI* region utilized (547 bp). Only one *B. celer* specimen was sequenced in this study, therefore precluding estimates of intraspecific variation. However, *B. celer* nested as an interior tree branch, suggesting that it can be used as a reference for the species.

Psyttalia lounsburyi and *P. humilis* are endoparasitoids of tephritids endemic to sub-Saharan Africa. The two species have been found in South Africa, Namibia, and

Kenya (Copeland *et al.*, 2004; Daane *et al.*, 2011; Mkize *et al.*, 2008; Rugman-Jones *et al.*, 2009), and both have been tested as exotic biocontrol agents of *B. oleae* in Europe and California, albeit with limited success (Borowiec *et al.*, 2012; Daane *et al.*, 2008). Previous studies of the genus *Psyttalia* based on *COI* sequences showed monophyly of *Psyttalia* species, including *P. lounsburyi* and *P. humilis* (Borowiec *et al.*, 2012; Cheyppe-Buchmann *et al.*, 2011; Schuler *et al.*, 2016), and phylogenetic reconstruction based on *COI* and 28S D2 sequences provided further support (Rugman-Jones *et al.*, 2009). Our NJ and ML analyses and the estimates of intra- and interspecific genetic distances support the morphological identification of the specimens analyzed in this study and the utility of standard DNA barcoding for the molecular identification of species belonging to the genus *Psyttalia*, at least for those with good intraspecific coverage.

Utetes africanus is a parasitoid reported in South Africa, Namibia, and Kenya (Copeland *et al.*, 2004; Daane *et al.*, 2011; Mkize *et al.*, 2008; Silvestri, 1913). It has been reported as being more abundant in wild olives than in cultivated olives (Caleca *et al.*, 2017; Giacalone, 2011; Mkize *et al.*, 2008; Neuenschwander, 1982), most likely because its short ovipositor is unable to reach fly larvae buried deep inside the pulp of the large fruit of cultivated olives. The genus *Utetes* was shown to be polyphyletic (Hamerlinck *et al.*, 2016), and three main clusters were recovered for *U. canaliculatus* with an exact correspondence between microsatellite genetic distances and a *COI* maximum parsimony tree (Hood *et al.*, 2015). Our NJ and ML trees also recovered non-monophyly for the genus, the same three *U. canaliculatus* clusters, and inconsistency between species designations and sequence clustering (e.g., *U. tabellariae* was positioned within *U. canaliculatus* in cluster 2). Comparison of genetic distances suggested that *U. canaliculatus* cluster 3 represents an evolutionary unit highly diverged from *U. canaliculatus* clusters 1 and 2. In agreement with the morphological identification, *U. africanus* was a monophyletic cluster with low intraspecific divergence (0.4%), thus supporting the use of these sequences as references for the species.

3.5.4 Chalcidoidea wasp species

Eupelmus spermophilus was found “emerging from the seeds of wild olive fruits” (Silvestri, 1915). This species was previously reported in Eritrea and the Western and the Eastern Cape provinces of South Africa (Mkize *et al.*, 2008; Silvestri, 1915). In agreement with previous phylogenetic analyses focusing on the *Eupelmus urozonus*

species complex (Al Khatib *et al.*, 2016; Al Khatib *et al.*, 2014), our NJ and ML trees showed concordance between monophyletic clustering and morphological identification for the genus *Eupelmus*, including *E. spermophilus*. Interspecific genetic distances were generally high, and supported the species designations. Interspecific divergence was exceptionally low for *E. urozonus*/*E. minozonus* (7.8%), suggesting a more recent divergence for this pair, represented as sister clades in the NJ tree. Maximum intraspecific genetic distances within this genus were exceptionally high for some species, suggesting the presence of cryptic diversity. For example, *E. annulatus* had a maximum intraspecific genetic distance of 8.7%, and the sequences were distributed between two well-supported clades composed by *E. annulatus* 1, 2, and 3 and *E. annulatus* 5, 6, and 7 (Figure. S4). The mean genetic distance between the two clades was 7.2%, and the maximum within clade distance was lower than 3.1%, thus suggesting that not all sequences designated as *E. annulatus* are conspecific. Although this was not the case for *E. spermophilus* (2.3%, this study), a pattern of high maximum intraspecific distances (4.0%–8.7%) was found for all the *Eupelmus* species reported in a previous assessment of this genus, except for *E. minozonus and E. gemellus* (2.7%) (Al Khatib *et al.*, 2014).

Eurytoma oleae and *E. varicolor* were reported to develop on the seeds of olives, and the species may be phytophagous seed wasps or parasitoids of seed wasps (Neuenschwander, 1982; Silvestri, 1915). Both species were previously found in Eritrea and South Africa (Western Cape) (Silvestri, 1915). *Eurytoma oleae* was also identified in a previous study in the Eastern Cape, as well as a *Eurytoma* sp. that most likely represented *E. varicolor* (Mkize *et al.*, 2008). The geographic range of *Eurytoma* species associated with olive trees probably extends to Kenya, where unidentified Eurytomidae were reportedly reared from wild olives (Copeland *et al.*, 2004). Our NJ and ML analyses recovered monophyletic clusters in accordance with species designations, including *E. oleae* and *E. varicolor*. As maximum intraspecific genetic distances in the genus *Eurytoma* ranged between 0.4% and 1.5%, future investigation of potential cryptic diversity in *E. oleae* using additional genetic markers may be warranted. The range of interspecific genetic distances (>10.2%) support the utilization of the standard barcoding *COI* region for species identification within this genus.

Sycophila aethiopica is possibly a parasitoid of seed wasps (Silvestri, 1915). The species was previously reported in Eritrea and South Africa (Western Cape)

(Neuenschwander, 1982; Silvestri, 1915), and most probably reported in the Eastern Cape as *Sycophila* sp. (Mkize *et al.*, 2008). *Sycophila aethiopica* was represented by a single sequence generated in this study, and none of the publicly available sequences were identified to the species level, therefore hampering estimation of intra- and interspecific divergences and specific NJ clustering. However, the single *S. aethiopica* sequence nested within the interior branches of the NJ and ML trees, thus supporting its utility as reference for the species. The genus *Sycophila* was shown to be monophyletic using nuclear markers (28S and 18S rRNA), and non-monophyletic using mitochondrial markers (16S and *COI*) (Chen *et al.*, 2004). The low statistical support for the deeper-level divergences in the NJ and ML trees also suggest that future phylogenetic reconstructions may have to include a combination of nuclear and mitochondrial sequences for the recovery of reliable branching patterns within this genus.

Neochrysocharis formosus (formerly *N. formosa*) is a non- specialized endoparasitoid with worldwide distribution, except for Australia (Chien & Ku, 2001). This species was also previously found in the Western Cape in several areas (including Paarl) (Neuenschwander, 1982), but it was not reported in the Eastern Cape (Mkize *et al.*, 2008). The classification of genera and species in the tribe Entedonini is controversial, particularly in the case of small-bodied species, such as *Neochrysocharis*. *Neochrysocharis* (*N. formosus* HM365028 and *N. clinias* HM365038) was previously shown to be, with respect to *Asecodes*, paraphyletic in molecular analyses, paraphyletic in combined (molecular and morphological) parsimony analysis, and monophyletic in combined Bayesian analysis (Burks *et al.*, 2011). The inclusion of the *N. formosus* Nf21 and *N. formosus* Nf18 sequences recovered the paraphyly of the genus *Neochrysocharis* with regards to *Asecodes*. Additionally, both the trees and the estimates of genetic divergence indicated that the *Neochrysocharis COI* sequence dataset is composed of two different species, with one species represented by *N. formosus* HM365028 and the other represented by *N. formosus* Nf21 and *N. formosus* Nf18 identified in this study. Deeper molecular coverage of species will be necessary to resolve taxonomic classifications within this group.

Ormyrus and similar species are considered to be parasitoids attacking seed wasps both in Eritrea and in the Western Cape (Silvestri, 1915), and no ormyrids are known to parasitize *B. oleae*. *Ormyrus* sp. was reportedly reared from wild and cultivated olives

in the Western and the Eastern Cape (Giacalone, 2011; Mkize *et al.*, 2008; Neuenschwander, 1982). Several attempts were made to obtain PCR products from *Ormyrus* sp. without success, suggesting that specific primers may have to be designed for future analysis.

3.5.5 *Wolbachia* and pseudogenes

Unintended amplification and sequencing of two types of fragments non-representative of the barcoding *COI* region occurred in some DNA extracts. One *Wolbachia* sequence, the most common bacterial endosymbionts in arthropods, was sequenced from *E. spermophilus*. This is known to occur when attempting PCR amplification of insect *COI* with universal primers from total genomic DNA (Smith *et al.*, 2012). Procedures for the quality control of the data (e.g., BLASTn searches) are mandatory to prevent false results in downstream assessments of genetic variation and phylogenetic reconstructions. Putative pseudogene fragments, possibly nuclear pseudogenes of mitochondrial origin (NUMTs), were also obtained with the genus-specific primers (Euryt-COI-F2/ Euryt-COI-R2) in three *E. varicolor* samples. This could be explained by the non-specificity of the primers for *E. varicolor*, as these were designed based on *E. oleae* sequences. PCR amplification of NUMTs is known to occur frequently in DNA barcoding of insects, and quality control for their identification (e.g., amino acid translation) can greatly contribute to detect and purge these sequences from *COI* datasets (Leite, 2012).

3.6 Conclusion

The present assessment of wasp species associated with wild and cultivated olives represents a comprehensive coverage of the rich endemic parasitoid and seed wasp diversity in South African olives. Sub-Saharan African Braconidae are particularly interesting due to their potential use as exotic biocontrol agents for controlling olive fly populations in regions where this pest lacks specialized natural enemies (e.g., California). The assemblage of Chalcidoidea associated with olives remains poorly studied, and details of the specific biology remain unknown for several species. For example, *E. spermophilus*, *E. oleae*, *E. varicolor*, *S. aethiopica*, and *Ormyrus* sp. have been variously reported as possible seed wasps, parasitoids of olive fruit flies, or hyperparasitoids. DNA analyses can be applied to the identification of immature insect life stages such as eggs, larvae, nymphs, or pupae, otherwise often impossible to

identify morphologically. This is particularly pertinent for the early detection of invasions, disseminations, and infestation outbreaks of agricultural pests. In the particular case of olives, methodologies inspired by DNA barcoding for species identification could also be used in the analyses of insect material collected from the interior of the fruits to elucidate the elusive lifestyle of the wasps and other insect groups associated with wild and cultivated olive trees.

Chapter 4: DNA-based identification of insect larvae gives new insights into the elusive lifestyles of olive seed wasps

4.1 Abstract

The olive industry in South Africa is relatively new and mainly focused in the Western Cape province, which has a predominantly Mediterranean climate. Some production losses in the region are due to the activity of olive seed wasps (OSW) associated with premature fruit drop. Wasps of the families Braconidae and Chalcidoidea associated with wild and cultivated olives were comprehensively described and barcoded in a previous study, but the status of several of these species as phytophagous, parasitoid or hyperparasitoid remains unclear. In this study, a PCR multiplex method based on reference *COI* sequences was developed for the simultaneous detection of five putative chalcid OSW (*Eupelmus spermophilus, Eurytoma oleae, Eurytoma varicolor, Neochrysocharis formosus* and *Sycophila aethiopica*) to identify immature specimens (e.g. larvae and pupae) collected from wild and cultivated olive seeds. Tests using reference DNA extracted from morphologically identified adults showed that the multiplex method was robust, sensitive, easily performed in a single PCR reaction, and produced unambiguous species-specific amplicons for the five species. The DNA-based identification tool using immature specimens collected from olive seeds confirmed the immatures to be *E. spermophilus*, *E. oleae* and *S. aethiopica*, proving they were phytophagous. This method can be used as a diagnostic tool for detection of pests as immatures, at the early stage of infestation when morphological identification is infeasible, and can provide an opportunity to gain new biological insights into the lifestyles of these elusive species. Future work will expand the survey over time and geographic range in order to capture the complete diversity of species and assess their potential associations.

4.2 Introduction

The cultivated olive is an ancient food crop thought to have been domesticated in the Mediterranean Basin, where it has been part of the agricultural landscape for at least 8,000 years (Rice *et al.*, 2003). More recently, the olive culture was disseminated from its original range to other world regions with similar climate such as California in North America, and the Western Cape province of South Africa. Californian olive orchards were relatively free from severe insect threats until the invasion and rapid expansion of the olive fruit fly (*Bactrocera oleae*) in 1998 (Rice *et al.*, 2003). As neither olive trees nor *B. oleae* are native

to North America, specialized natural enemies are non-existent in the region and control measures are now routinely performed. South African olive growers face a different set of challenges. Cultivated olives of European origin (*Olea europaea* subsp. *europaea*) were brought in during the early colonial period of the Western Cape, a region where the African wild olive (*Olea europaea* subsp. *cuspidata*), a relative of the cultivated olive, is frequently found in the Afromontane landscape that stretches vast areas of Southern and Eastern Africa, and further into Asia. In the Western Cape, wild olives are host to a particularly rich assemblage of specialized olive fruit fly species that includes *B. oleae* and *Bactrocera biguttula*, olive lace bugs, olive flea beetles, olive seed wasps and parasitoids (Mkize *et al.*, 2008; Powell *et al*., 2019; Teixeira da Costa *et al*., 2019). This well-adapted entomofauna most likely co-evolved with the African wild olive and was established before the advent of commercial olive production. Therefore, the introduction of cultivated olives into the region provided the native species with an opportunity to utilize a new host with a high degree of similarity to the native wild host.

In contrast to other olive-producing regions, *B. oleae* is currently not a major concern in the Western Cape. The nonthreatening status of the olive fruit fly was hypothesized to be a result of the activity of the specialized native enemies, predominantly *Psyttalia humilis, Psyttalia lounsburyi, Utetes africanus* and *Bracon celer* that parasitize the larvae of *B. oleae* growing within the flesh of olives (Copeland *et al.*, 2004; Neuenschwander, 1982; Silvestri, 1913, 1914, 1915). Recently, climatic differences have been suggested to play an important role in the relatively low infestations levels of cultivated olives by *B. oleae* (Caleca *et al.*, 2017; Caleca *et al.*, 2015; Giacalone, 2011). Olive production losses in the Western Cape occur to some extent, but are mainly due to the action of olive seed wasps (generically designated as OSW) feeding on the olive kernel during the early stages of development, which often results in the premature drop of the fruit (Allsopp, 2017). Olive seed wasps are not known to occur in other olive-producing world regions, and are currently poorly characterized.

Surveys conducted in sub-Saharan Africa have reported the presence of a broad complex of wasps associated with wild and cultivated olives, including species endemic to the region (Hoelmer *et al.*, 2011). A previous survey reported species belonging to the family Braconidae and superfamily Chalcidoidea in the Western Cape, of which four braconids and five chalcids (Powell *et al.*, 2019). The family Braconidae is large and species-rich containing more than 19,000 described species, and is composed by highly specialized parasitoids

(Dowton, 1999; Dowton *et al.*, 2002; Li *et al*., 2016; Shi *et al.*, 2005; Zaldivar-riverón *et al.*, 2006). The superfamily Chalcidoidea (Hymenoptera) is composed of very small wasps with an estimated diversity of 500,000 morphologically distinct species that include parasitoid and phytophagous groups (Munro *et al.*, 2011).

Great focus has been centred around parasitoid wasps of the family Braconidae, especially species endemic to sub-Saharan Africa, as they hold the potential to be utilized as exotic biological control agents in regions where the olive fruit fly causes significant damage (Copeland *et al*., 2004; Hoelmer *et al*., 2011; Mkize *et al*., 2008; Silvestri, 1913). Chalcid wasps associated with olives, on the other hand, have attracted less attention due to their modest impact on international olive production. These wasps include *Eupelmus spermophilus* Silvestri, *Eurytoma oleae* Silvestri, *Eurytoma varicolor* Silvestri, *Sycophila aethiopica* (Silvestri), *Neochrysocharis formosus* (Westwood), and *Ormyrus* sp. (Westwood). The status of these species as phytophagous, parasitoid or hyperparasitoid is not fully clarified.

Eupelmus spermophilus is known to feed on the seeds of olives (Caleca *et al.*, 2019), and *N. formosus* has been described as a non-specialized parasitoid (Saleh *et al.*, 2010; Luna *et al.*, 2011). *Eurytoma oleae* is frequently found emerging from olives and is considered as a potential OSW; however, it has also been suggested to be a parasitoid of olive seed-feeders or hyperparasitoid of other wasps (Silvestri, 1915). *Eurytoma varicolor* and *S. aethiopica* also found emerging from olives, are not known to parasitize olive fruit flies (Mkize *et al*., 2008; Powell *et al*., 2019). In previous surveys, unidentified *Ormyrus* species (Ormyridae) were collected from areas in the Eastern and Western Cape but the status regarding their lifestyles remains unknown (Mkize *et al*., 2008; Powell *et al*., 2019). However, species such as *Ormyrus striatus* (Silvestri) have previously been reared from cultivated olives in South Africa and is considered to be parasitoids of seed wasps (Neuenschwander, 1982; Silvestri, 1915).

The identification of the species of insect larvae found within olive seeds could provide direct insight into the lifestyle of these species, generating useful information for the development of pest control strategies in the Western Cape. As such, the first objective of this study was to perform species identification of immature insect specimens collected from within wild and cultivated olive seeds to elucidate the lifestyles of *Eupelmus spermophilus, Neochrysocharis formosus, Eurytoma oleae, Eurytoma varicolor* and *Sycophila aethiopica* using a species-specific multiplex PCR. In addition to developing a multiplex PCR for

identification of immature insect specimens, the multiplex PCR provided a tool for the elucidation of the lifestyles of five elusive chalcid wasp species associated with wild and cultivated olives in the Western Cape province of South Africa. This multiplex PCR tool can be used to screen specimens collected from a sample of olives suspected to be infested with olive seed wasp that would firstly, confirm the presence or absence of OSW and secondly, identify the OSW species present.

4.3 Materials and methods

4.3.1 Sample collection and DNA extraction

Immature wild (n = 300) and cultivated olives (n = 9,397) were collected from three sites in two locations in the Western Cape during the South African fruiting season of 2018 (Table S11). The collected olive fruit were manually inspected for signs of parasitism i.e. exit holes from olive fruit flies or olive seed wasps. Female olive fruit flies feed on the juice of developing olive fruit in order for their ovaries to mature therefore, they puncture the skin of the olive with their ovipositors and suck the juice from the olive pulp through the puncture hole. These puncture holes rapidly close to prevent oxygen and microorganisms from entering the fruit and degrading the olive pulp, which then become challenging to detect during processing of olive fruit. Thus, the only sign of parasitism is the exit holes of fully developed olive fruit fly larvae, which are large enough for oxygen and microorganisms to enter the fruit and consequently destroy the pulp and oil content. In the case of olive seed wasps, the females lay eggs using her ovipositor early in the fruiting season while the olive is small, and the pip is soft. The narrow tunnel left by the ovipositor supplies oxygen to the developing egg. The hatched larvae feed on the seed until the final instar where it develops into a pupa. An adult wasp emerges from the pupa and chews an exit hole from the seed through the pip and flesh where it exits.

The exit holes of adult olive seed wasps are smaller than olive fruit fly larvae and have well-defined edges containing a tunnel originating straight from the olive seed (Caleca *et al*., 2015). As a result, olive fruit with or without exit holes were manually inspected to increase the probability of detecting the presence of larvae or pupae that may have become trapped within the seed during pit hardening. For the recovery of immature insect specimens from the interior of the seeds, the fruits were carefully cut open using a 3 – 30 mm plumber's pipe cutter to crack the hard pip of the olive fruit. The open pip exposed the seed and was visually inspected for the presence of larvae and pupae within the seed structure (Figure 4.1). Larvae

and pupae (n = 50) were manually isolated from within the seeds and individually stored in absolute ethanol at 4°C until DNA extraction. Total DNA was extracted from each specimen following a standard phenol-chloroform method (Sambrook *et al.*, 1989). Nucleic acid concentration (ng/µL) and purity of the extracts were assessed using NanoDrop 2000/2000c Spectrophotometer.

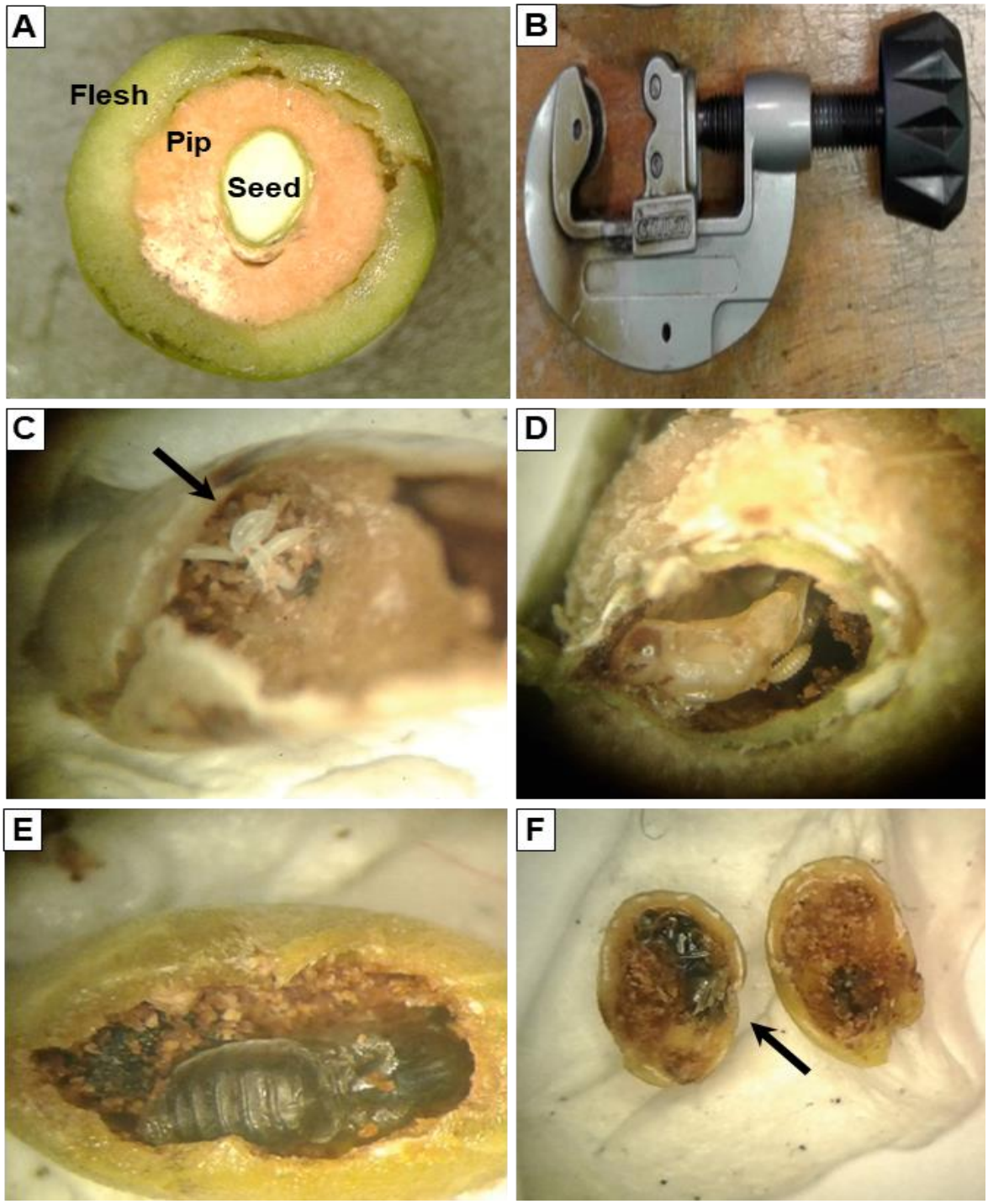

Figure 4.1. Examples of manual inspections of wild and cultivated olive seed structures. (A) Cross-section of an olive; (B) 3 – 30 mm pipe cutter; (C) Eggs found within the olive seed structure; (D) Small larva and unknown pupa within the olive seed structure; (E) Unknown pupa within the olive seed structure; (F) Adult wasp trapped inside the olive seed structure.

4.3.2 PCR multiplex for the identification of OSW

Species-specific primers were designed for each putative OSW species (*E. spermophilus, E. oleae, E. varicolor, N. formosus* and *S. aethiopica*) based on reference barcoding (*COI*) sequences generated in a previous study (Powell *et al.*, 2019). Although the status of *Ormyrus* sp. is currently uncertain, no reference sequences are currently available for the design of species-specific primers; therefore, the species was excluded from this work. All sequences available for each target species were aligned using the MAFFT algorithm (Katoh & Standley, 2013) implemented in Geneious Prime (www.geneious.com) (Kearse *et al.*, 2012). The total alignment was manually checked for adequate regions for the design of species-specific primers that theoretically annealed to the target DNA but not to any of the other species. One species-specific primer pair was designed for each species except for *E. spermophilus* and *E. varicolor,* for which the reverse primer is shared. Preliminary singleplex PCRs were performed for the confirmation of the expected amplicons using reference DNA extracted from adult specimens representative of each species. Singleplex amplifications were tested using unlabelled and fluorescently labelled primers with the same sequence, followed by fragment size detection by agarose gel and capillary electrophoresis, respectively.

The multiplex was designed to generate species-specific PCR products with non-overlapping fragment sizes between 100 bp and 500 bp, allowing for the simultaneous amplification and detection either in agarose gel or in capillary electrophoresis (Table 4.1). All PCR amplifications were performed in 5 µL total volume using 1X QIAGEN® Multiplex PCR kit (QIAGEN), 0.2 µM of each primer and 0.5 µL template DNA. Thermocycling conditions were as follows: initial denaturation at 95 °C for 15 min; 28 cycles at 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min. The larvae and pupae collected from olive seeds were individually analysed in multiplex PCR with fluorescently labelled primers for automatic fragment size detection by capillary electrophoresis, under the same conditions (Table 4.1). Capillary electrophoresis was performed using LIZ600 as an internal size standard, at the Central Analytical Facilities of Stellenbosch University.

Table 4.1. List of species-specific primers used in the multiplex PCR amplification of putative olive seed wasps found within the seeds of wild and cultivated olives in the Western Cape province of South Africa. *Reverse primer shared between *E. spermophilus* and *E. varicolor*.

Species	Primer name	Primer (5´- 3´)	Amplicon size (bp)
Neochrysocharis formosus	FOR-F2	TAT TTA TTG GGT CGG GGA CG	130
	FOR-R	ATA ATA GAA CTG ACC CCC GC	
Eupelmus spermophilus	EUP2-F	CTC CAT ATT GCG GGG GCT TC	154
	EUP-R*	CTC CAG CTA ATA CAG GAA GTG	
Sycophila aethiopica	SYC-F	ATG CCT GTA ATA ATA GGA GGG	208
	SYC-R	CCC CAT GAG ATA TAT TAC CAG	
Eurytoma oleae	EUOL-F	TTG TTA CAA CTC ATG CTT TTG TA	310
	EUOL-R	CCT ATA ATT GAA CTT ACC CCC	
Eurytoma varicolor	EUVAR-F	GAT TTG GAA ACT TCT TAA TTC CA	378
	EUP-R*	CTC CAG CTA ATA CAG GAA GTG	

4.4 Results

This work builds upon a previous survey of wasps found emerging from wild and cultivated olives in the Western Cape that generated reference DNA sequences representative of the species identified (Powell *et al.*, 2019). The first objective of this study was to perform species identification of immature insect specimens (e.g. larvae and pupae) collected from within olive seeds to elucidate the lifestyle of *E. oleae, E. varicolor, E. spermophilus, N. formosus*, and *S. aethiopica* using a multiplex PCR composed by species-specific primers for each species. A larger number of wild olive fruits was analysed by comparison to cultivated olives, as the first seem to be more frequently infested by a wider diversity of wasp species (Mkize *et al.*, 2008; Powell *et al*., 2019; Silvestri, 1915). In addition to developing a multiplex PCR that allows for species identification of immature insect specimens, the multiplex PCR provided a tool for the elucidation of the lifestyles of elusive chalcid wasp species, previously unknown or undetermined. The multiplex PCR was designed to survey immature insect specimens within the known species panel for which reference sequences were available. Although other species are found in association with olive fruits in South Africa, they either feed on the flesh of olive fruits (*B. oleae* and *Bactrocera biguttula*) or are known parasitoids of olive fruit flies (*P. humilis, P. lounsburyi, B. celer* and *U. africanus*). Therefore, these species were excluded from the multiplex as they are unlikely to be found within the olive seed structure. The size of the amplicons was designed to fall in the range between 100 bp and 500 bp to increase the potential co-amplification of the low amounts of DNA expected to be isolated from small OSW specimens potentially parasitized by other

species (Figure 4.2).

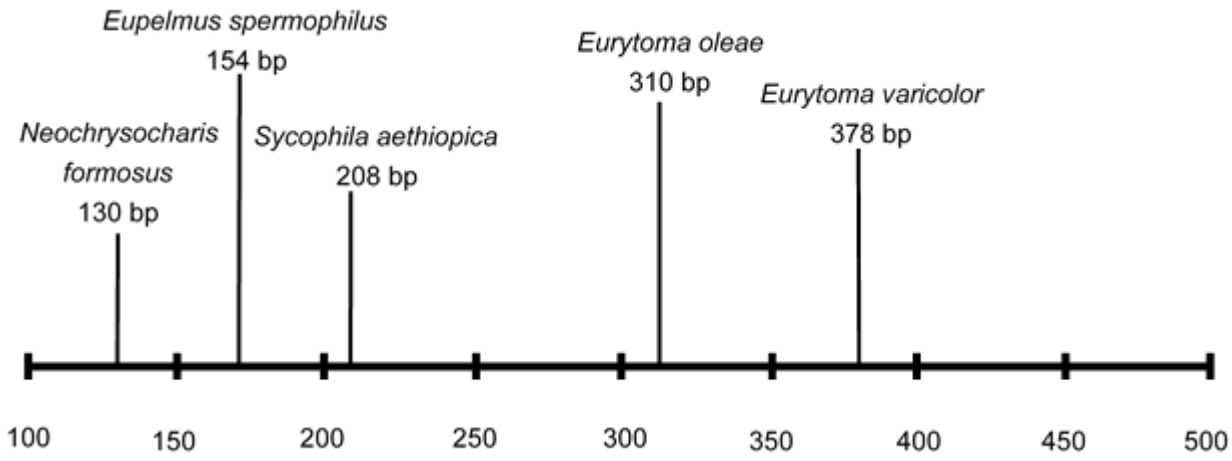

Figure 4.2. Expected fragment sizes (bp) of the species-specific multiplex PCR products for the identification of five putative olive seed wasp species: *Neochyrsocharis formosus*, *Eupelmus spermophilus*, *Sycophila aethiopica*, *Eurytoma oleae and Eurytoma varicolor***.**

4.4.1 DNA recovery from immature OSW

The size of the OSW specimens collected from the interior of olive seeds varied between 1.23 mm and 3.53 mm. Species identification based on morphological characters prior to DNA extraction was not attempted, as diagnostic characters for these species are either absent or unreliable for the immature stages. Total DNA was successfully isolated from all specimens, and ranged between 2.30 ng/µL and 42.65 ng/µL.

4.4.2 Species-specific PCR multiplex

The present method includes species-specific primers for five wasp species that putatively develop within olive seeds. The preliminary singleplex PCR tests using reference DNA samples from identified adult specimens showed the expected fragment size in both in agarose gel and capillary electrophoresis methods of detection (Figure 4.3). Preliminary multiplex PCR tests using the reference DNA for each species also showed species-specific amplification of the expected fragments both in agarose gel and capillary electrophoresis (Figure 4.4). Non-specific amplification was detected in the *S. aethiopica* reference sample. Following the preliminary singleplex and multiplex PCR tests, individual immature insect samples collected from within olive seeds were tested using the multiplex PCR (n = 50). All samples analysed were successfully assigned to a species based on amplicon size detected

in both agarose gel and capillary electrophoresis (Figure 4.5). Non-specific PCR products were detected in two samples of *S. aethiopica* (Figure 4.5b).

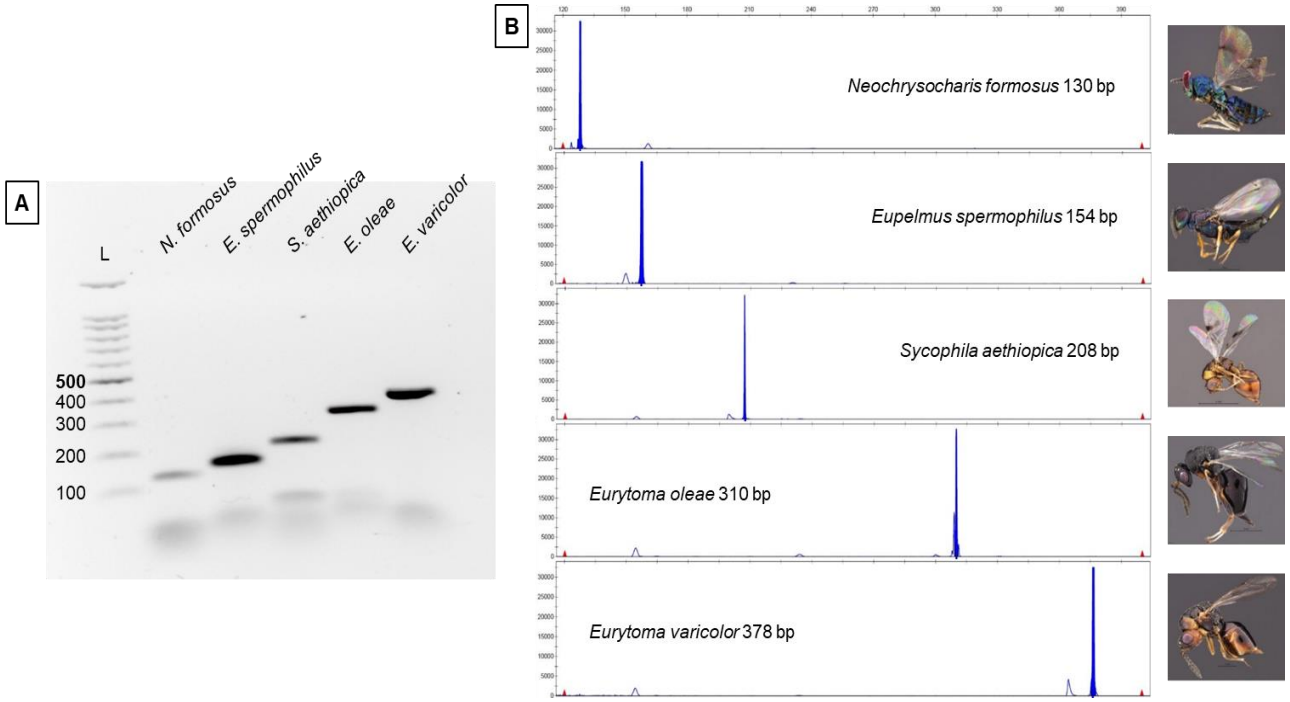

Figure 4.3. Singleplex PCR amplification of reference DNA extracted from morphologically identified adult specimens of five putative olive wasp species associated with wild and cultivated olives in the Western Cape province of South Africa, using species-specific primers. (A) Detection of the fragment size of the species-specific PCR products on a 1.5% agarose gel. (B) Detection of the fragment sizes of the species-specific PCR products using capillary electrophoresis.

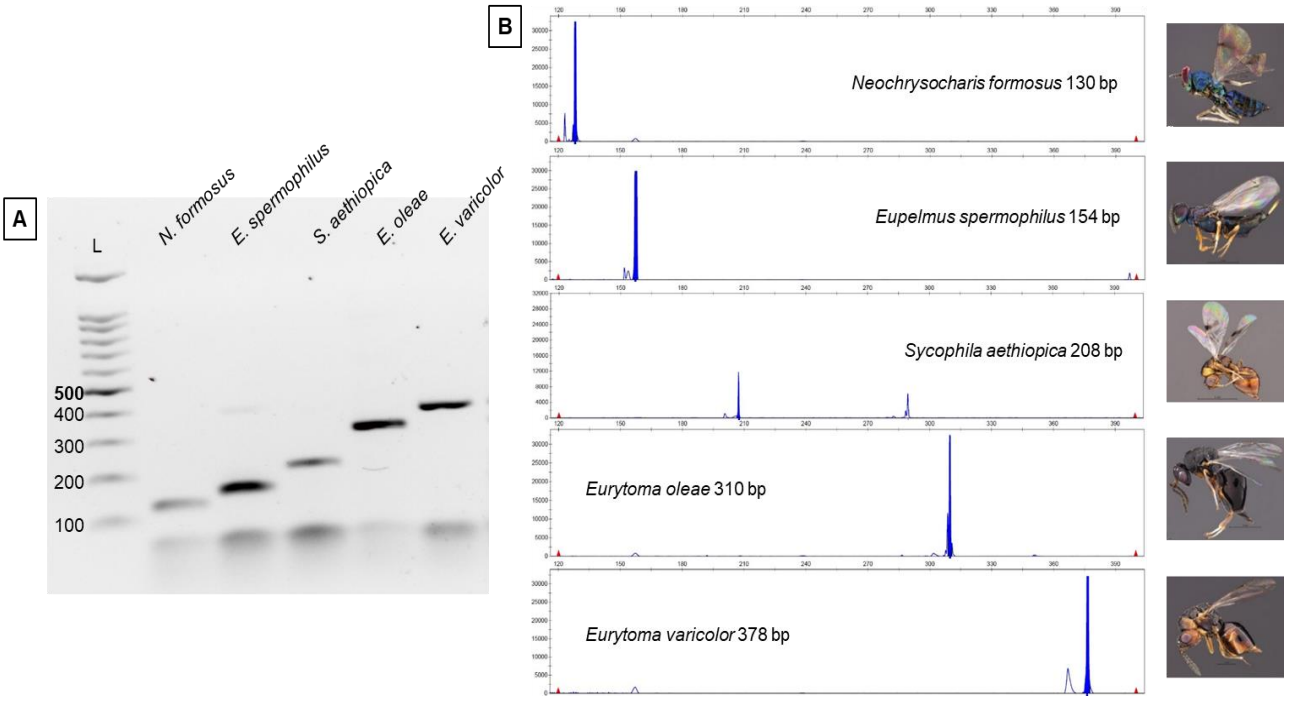

Figure 4.4. Multiplex PCR amplification of reference DNA extracted from morphologically identified adult specimens of five putative olive wasp species associated with wild and cultivated olives in the Western Cape province of South Africa, using species-specific primers. (A) Detection of the fragment size of species-specific PCR products on a 1.5% agarose gel. (B) Detection of the fragment size of the species-specific PCR products using capillary electrophoresis.

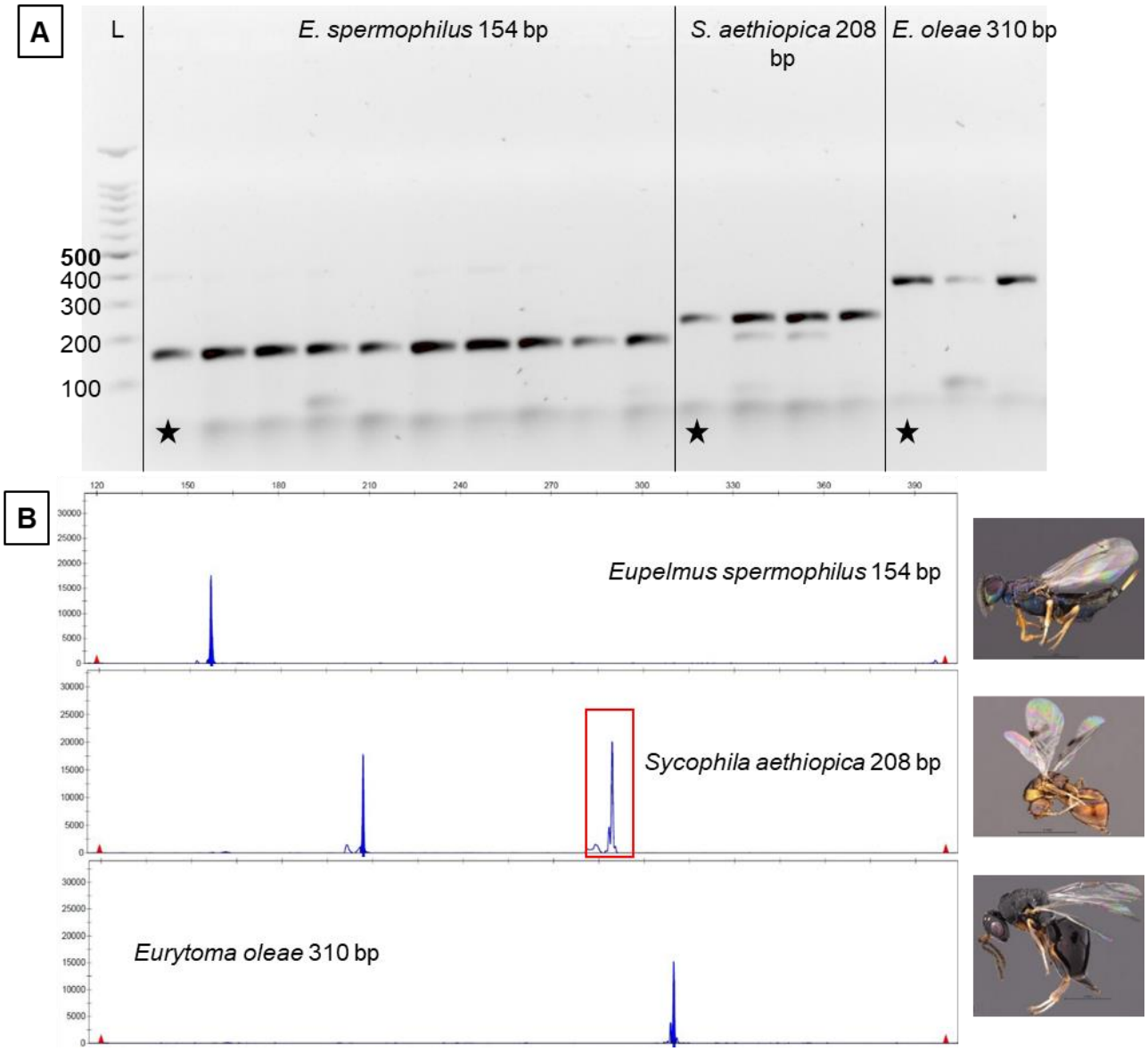

Figure 4.5. Multiplex PCR amplification of larvae and pupae samples found within the seeds of wild and cultivated olives in the Western Cape provinces of South Africa. (A) Detection of fragment size of the species-specific PCR products on a 1.5% agarose gel. Stars indicate the reference DNA sample for that particular species. (B) Detection of the fragment size of the species-specific PCR products using capillary electrophoresis. The red box indicates a nonspecific PCR product associated with *Sycophila aethiopica*.

The majority of the total larvae and pupae samples were identified as *E. spermophilus* (90%) (Figure 4.6). All of the OSW found in cultivated olives were *E. spermophilus*, whereas wild olives also were infested by *S. aethiopica* and *E. oleae*.

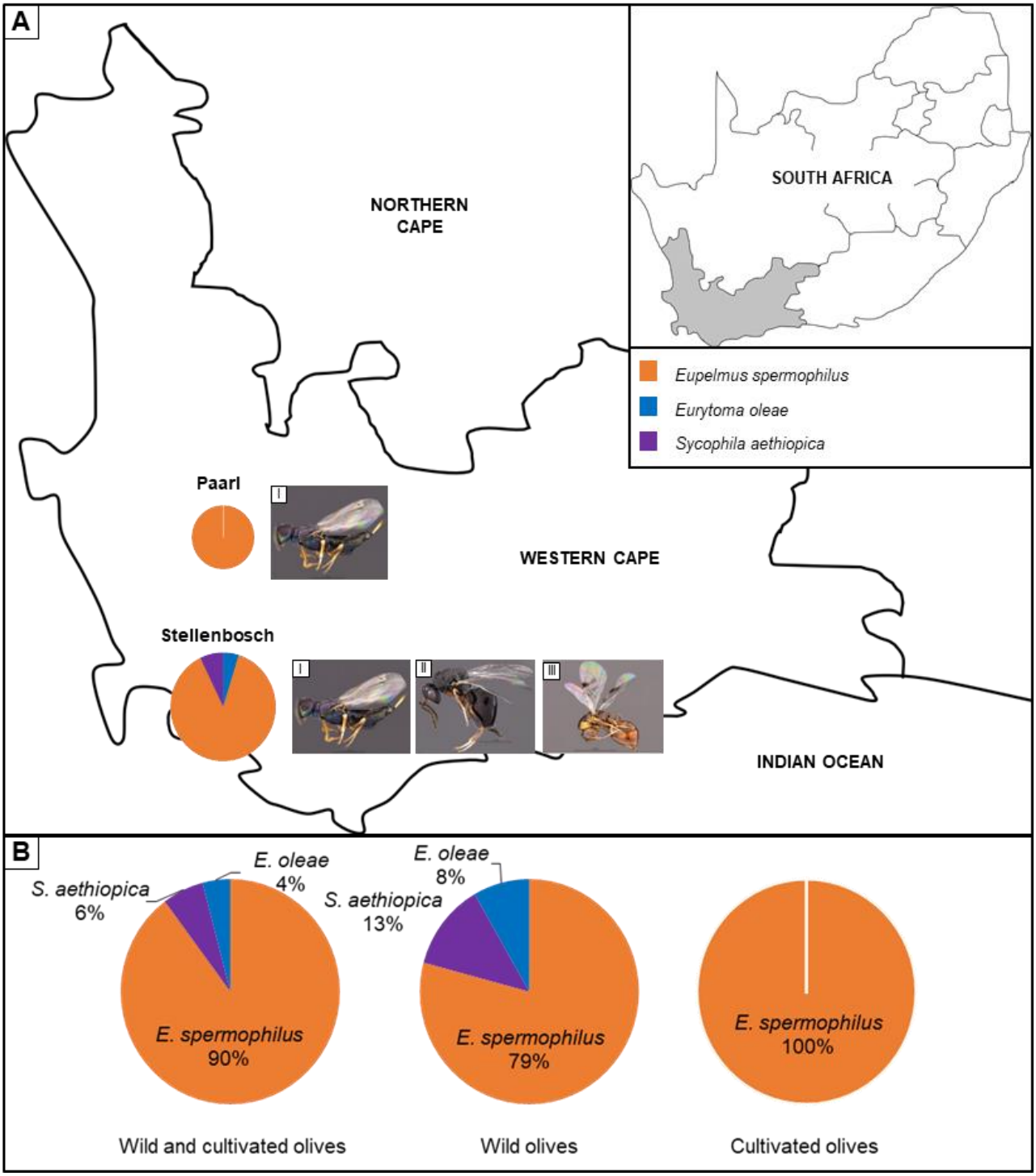

Figure 4.6. Areas of collection of wild and cultivated olives in the Western Cape province of South Africa and the relative proportions of the olive seed wasp species at each collection area. (A) Pie charts represent the relative proportions of Chalcidoidea species recovered from within olive seeds collected in each area. The size of the circles is proportional to the total number of larvae and pupae specimens recovered from each area. Images represent the species found at each collection area; (I) *Eupelmus spermophilus,* (II) *Eurytoma oleae,* (III) *Sycophila aethiopica*. (B) Relative proportions of *Eupelmus spermophilus, Sycophila aethiopica* and *Eurytoma oleae* identified within seeds of wild and cultivated olives collected at three sites in the Western Cape province of South Africa: Olyvenbosch Olive Farm (Paarl), Waterford Wine Estate (Stellenbosch) and Agricultural Research Council Nietvoorbij (Stellenbosch).

Eurytoma oleae represented only 4% of the samples (n = 2), and was exclusively found within the seeds of wild olives. In our previous survey, *E. oleae* represented 7% of the total adult wasps and it was reared exclusively from wild olives (Powell *et al.*, 2019). *Eurytoma varicolor* was not detected in this study; however, the species represented 16% of the total adult wasps exclusively reared from wild olive fruit from five areas in the Western Cape province of South Africa in our previous survey (Powell *et al.*, 2019). Of the five areas surveyed, *Eurytoma varicolor* was abundant in Somerset West (11%), after Stellenbosch (85, 3%) and least abundant in Stanford (0.74%). Within Stellenbosch, six different sampling sites were surveyed including Coetzenburg Athletics Stadium (50%), Nonke Plant Nursery (33, 6%) and Waterford Wine Estate (4, 3%), the latter included in the present study. In the present survey, *S. aethiopica* represented 6% of the samples and it was also exclusively found within the seeds of wild olives. In our previous survey, *S. aethiopica* also represented 6% of the adult wasps reared; however, the sampling area was wider and included 16 different locations, whereas the present study only included two locations. *Neochrysocharis formosus* was not detected in this study; however, it represented the lowest proportion (3.6%) of chalcid wasp species in our previous survey.

4.5 Discussion

4.5.1 DNA recovery and species-specific PCR multiplex

Despite the small size of some specimens (average = 1.71 mm), all extracts yielded workable amounts of DNA for PCR amplification. Species-specific multiplex PCR is a relatively simple and inexpensive technique that can be used in a wide range of biological materials, and has proven particularly useful when screening for the presence of more than one species in the same sample (Gonçalves *et al.*, 2012). The multiplex allowed for species identification even in DNA extracts with low concentration (<10 ng/µL), as was the case of 40% of the samples. Non-specific amplification was detected in two larvae samples identified as *S. aethiopica* using the multiplex PCR. This was expected as the same non-specific amplification was present in the preliminary multiplex PCR testing of the reference *S. aethiopica* sample. Furthermore, the non-specific PCR products of the preliminary multiplex test was not detected in agarose gel but rather in capillary electrophoresis, which may be a result of agarose gels that do not offer the high sensitivity and resolution compared to capillary electrophoresis (Gupta *et al.*, 2010). Additionally, the reference DNA barcoding sequences used to design species-specific primers were only ~650 bp in length thus limiting the design of primers and the possibility of amplifying undesired fragments. Moreover, these

sequences are fairly similar containing only a few polymorphic sites representative of each species.

4.5.2 New insights into the lifestyles of putative OSW species

Nine wasp species are known to be associated with wild and cultivated olives, of which four Braconidae (*B. celer, P. humilis, P. lounsburyi* and *U. africanus*) are known to be parasitoids of the olive fruit fly *B. oleae,* and possibly of *B. biguttula*. Of the remaining five Chalcidoidea, only *E. spermophilus* was a confirmed OSW, and the status of *N. formosus, E. varicolor, E. oleae* and *S. aethiopica* remained unclear (Silvestri, 1915). In a previous survey investigating the insects associated with wild and cultivated olives in the Western Cape province of South Africa, *E. spermophilus* was the most abundant chalcid wasp species recovered and the most abundant species detected in the present study. Contrastingly, a survey conducted in the Eastern Cape province of South Africa found *E. spermophilus* at low rates of infestation suggesting that *E. spermophilus* may be the primary OSW causing damage in the Western Cape but not in the Eastern Cape (Mkize *et al.*, 2008).

Eurytoma oleae and *E. varicolor* have been reported to develop inside the seeds of olives, and also suggested that the species could potentially be phytophagous seed wasps or parasitoids of seed wasps (Neuenschwander, 1982; Silvestri, 1915). In our previous study, *E. oleae* was exclusively reared from wild olives and most abundant in Riversdale (Infestation rate = 1.89 %), a sampling site not included in the present study. In the present survey, *E. oleae* only represented 4 % and was also only found in wild olives hence, wild olives seem to be the preferred host of *E. oleae,* and potentially the exclusive host of the species. As the status of *Ormyrus* sp. is currently unknown and was excluded from the multiplex panel, *E. oleae* could potentially be a parasitoid of *Ormyrus* sp. or vice versa. In order to obtain reliable results, sampling efforts should include Riversdale, where *E. oleae* was most abundant and reference sequences and primers should be designed for *Ormyrus* sp. *Eurytoma varicolor* may not have been detected as only immature insect samples from two locations where this species was not previously found in abundance were analysed, thus potentially limiting the probability of detecting its presence. To increase the probability of detecting *E. varicolor*, the sampling areas should include Coetzenburg Athletics Stadium and Nonke Plant Nursery from Stellenbosch and Somerset West included in our previous survey (Powell *et al.*, 2019).

Sycophila aethiopica was reported to have been reared from olives infested by *B. oleae;* however, the species was suggested to be not a parasitoid of the olive fruit fly but rather a seed feeder or a parasitoid of seed feeders (Silvestri, 1915). *Sycophila aethiopica* was exclusively found in wild olives both in the previous survey and the present study suggesting that the species prefers or is exclusive to wild olives. Furthermore, this study provides the first evidence of *S. aethiopica* as an olive seed wasp.

Neochrysocharis formosus, formerly known as *N. formosa,* is a non-specialized endoparasitoid reported to have emerged from wild and cultivated olives infested with *B. oleae* in South Africa (Neuenschwander, 1982). Moreover, *N. formosus* seems to be a particularly rare species as it was previously only found in one location (Paarl). Although the same location was included in the present study, a much larger number of olives may have to be analysed for obtaining evidence of the presence of *N. formosus* inside the seed of the fruit.

Confirmation of the OSW status of *E. varicolor* and *N. formosus,* which were not detected in this survey, will require a more comprehensive sampling strategy following the distribution of wasp species observed in our previous survey. Moreover, publicly available sequences are presently restricted to the standard region (~650 bp) of the 5´-end of the mitochondrial cytochrome oxidase I gene (*COI*) and partial 28S ribosomal RNA (rRNA) sequences of *E. spermophilus* (*COI* n = 11)*, E. oleae* (*COI* n = 9)*, E. varicolor* (*COI* n = 6)*, S. aethiopica* (*COI* n = 1)*,* and *N. formosus* (*COI* n = 2; 28S rRNA n = 17), most of which were generated in our previous study. The available sequences are inadequate for the design of reliable species-specific primers due to the short length. Consequently, sequencing the complete mitochondrial genomes of these species would allow for the detection of more polymorphic sites specific to each species thus eliminating potential non-specific amplification in multiplex PCR.

4.6 Conclusion

We surveyed immature insects found within the seed structure of wild and cultivated olives using a species-specific multiplex PCR. The multiplex performed well in reference DNA extracted from adult specimens and in a variety of larvae and pupae of various sizes, showing high-sensitivity and species-specificity. The range of PCR products allowed for unambiguous species identification using either agarose gels and/or capillary electrophoresis. The flexibility in the detection step of the amplicons is a significant advantage of the method, as identification of OSW can be affordably performed by laboratories who cannot rely on access to sequencing facilities. This study provides new insights into the lifestyles of *S. aethiopica* and *E. oleae* as OSW, and confirms *E. spermophilus* as a seed feeder. The status of *E. varicolor* and *N. formosus* as OSW was not confirmed. These results can assist in the early detection and control of OSW, the main cause of agricultural losses in the olive industry in the Western Cape of South Africa. Additionally, this study advances the present status of species previously not known as OSW and that have remained undetermined since the early 1900's. In the future, this multiplex PCR can be expanded to include other species associated with wild and cultivated olives, as the size gaps between the existing amplicons allow for inclusion of additional PCR products, if new adequate species-specific primers are designed.

Chapter 5: The mitochondrial genomes of *Psyttalia concolor, Psyttalia humilis*, and *Psyttalia lounsburyi*, three parasitoids of the olive fruit fly

5.1 Abstract

Braconidae wasps belong to the second largest family within Hymenoptera, and are interesting due to their potential as biocontrol agents of olive fly populations in regions where this pest lacks specialized native enemies. Three parasitoid species, *Psyttalia* concolor, *Psyttalia humilis* and *Psyttalia lounsburyi* (Braconidae: Opiinae) are important candidates for biocontrol applications of the olive fruit fly particularly *P. humilis* and *P. lounsburyi,* as they are endemic endoparasitoids of olive fruit flies in sub-Saharan Africa. Despite the relevance of these two species in South African wild and cultivated olive ecosystems, genetic data is presently limited to *COX1* (DNA barcoding) sequences. This study aimed at contributing to an improved phylogeny of the group by generating the new mitogenomes for *P. concolor, P. humilis* and *P. lounsburyi*. Near-complete mitochondrial genomes of *P. humilis* and *P. lounsburyi* were recovered using the NGS Ion Torrent Platform at Stellenbosch University. The sequences were assembled and annotated using *Psyttalia concolor*, sequenced through Sanger sequencing, as a reference sequence, and compared to other Braconidae sequences. The sequence coverage for *P. humilis* and *P. lounsburyi* was 2190.4x and 80.7x respectively. The sequences of *P. concolor* (14,859 bp) *P. humilis* (14,861 bp) and *P. lounsburyi* (14,797 bp) displayed the typical metazoan gene content; however, the non-coding AT-rich region will need further confirmation. The *Psyttalia* species had identical gene arrangements; however, they differed from other publicly available Braconidae mitogenomes, with all rearrangements involving tRNAs. Phylogenetic reconstruction of the family Braconidae confirmed the division of cyclostome/non-cyclostome wasps. Furthermore, the subfamily Aphidiinae was verified as a sister group to the cyclostome wasps, a group previously considered as a separate family (Aphidiidae). In addition to recovering the new near-complete mitogenomes of three important parasitoid wasp species, this study advances comparative mitogenomics of Braconidae and contributes to the complex phylogeny, a group in which gene rearrangements are frequent.

5.2 Introduction

The family Braconidae is a large and species-rich taxonomic group that includes 40 subfamilies represented by over 1,000 genera, and more than 19,000 described species (Shi *et al.*, 2005; Yu *et al.*, 2012). Braconidae is mostly composed of highly specialized parasitoids, and the majority of the subfamilies is either ectoparasitic idiobiont (i.e. the host is unable to recover after the paralysis induced by the ovipositing wasp), or endoparasitic koinobiont (i.e. the host can recover after oviposition and develop normally, completing the larval instars) (Dowton, 1999; Dowton *et al.*, 2002). In general, the family exhibits host-specific relationships at the subfamily level; however, this is less true for ectoparasitoids (Dowton *et al.*, 2002; Shi *et al.*, 2005). For example, the endoparasitic Microgastrinae attack only lepidopteran larvae, with the exception of one species associated with Trichoptera (van Achterberg, 2002), and the endoparasitic Helconinae parasitize coleopteran larvae. In contrast, the ectoparasitic Braconinae attack a variety of holometabolous larvae, and the subfamily has been used as a model for studying the evolutionary transition between ecto- and endoparasitism (Dowton *et al.*, 2002; Shi *et al.*, 2005). The family Braconidae is divided into two major groupings of subfamilies, the cyclostomes and the non-cyclostomes. Cyclostomes are discriminated by a cavity above the mandible (hypoclypeal depression), a synapomorphy of the group, and comprise all the ectoparasitoids as well as some endoparasitoids, and all known phytophagous braconids (Dowton, 1999; Shi *et al*., 2005). The cyclostome complex has been reported as monophyletic based on morphology (Gauld, 1988; Wharton *et al*., 1992), with the remaining non-cyclostome subfamilies as a sister clade based on integrated molecular and morphological data (Zaldivar-riverón *et al.*, 2006). Molecular studies using the mitochondrial 16S and the nuclear 28S rDNA gene regions also recovered cyclostomes as monophyletic (Belshaw *et al.*, 1998; Belshaw & Quicke, 1997; Dowton *et al.*, 1998). However, despite previous studies having provided extensive taxon coverage of Braconidae species, none were able to resolve phylogenetic relationships within cyclostomes with high statistical support (Dowton, 1999). Despite the family Braconidae having received considerable taxonomic attention in recent years, substantial confusion still persists over the definitions of several subfamilies, especially among the cyclostomes (Zaldivar-riverón *et al.*, 2006).

The olive fruit fly, *Bactrocera oleae* (Rossi), is well-known as a major historical pest of cultivated olives in the Mediterranean Basin. More recently, it also became a threat in California and Iran, where it quickly spread after the invasions were first detected in 1998

(Rice *et al.*, 2003) and 2003, respectively (Jafari & Rezaei, 2004; Ramezani *et al.*, 2015). Currently, insecticides remain the principal method of olive fruit fly control; however, their success is limited and they are environmentally harmful (Daane & Johnson, 2010; Hoelmer *et al.*, 2011). Efforts for finding and implementing effective agents for the biocontrol of the olive fruit fly started over 100 years ago, and are still underway (Kapatos *et al.*, 1977; Liaropoulos *et al.*, 1977; Liotta & Mineo, 1968; Monastero, 1931; Monastero & Delanoue, 1966; Raspi, 1995; Raspi & Loni, 1994; Silvestri, 1913, 1914, 1939). For example, California introduced an importation program for parasitoids wasps as natural enemies of *B. oleae* in 2003, and surveys for Braconidae associated with olive fruit flies have been conducted in South Africa, Namibia, Kenya, La Réunion, Canary Islands, Morocco, Pakistan, India and China (Daane *et al.*, 2011). The majority of olive fruit fly parasitoids were recovered from surveys of wild olives (*Olea europaea* L. subsp. *cuspidata*) in sub-Saharan Africa, the most likely the region of origin of *B. oleae* (Nardi *et al.*, 2010, 2005; Teixeira da Costa *et al.*, 2019). The Californian program focused on the evaluation and release of *Psyttalia humilis* (Silvestri) and *Psyttalia lounsburyi* (Silvestri), two koinobiont endoparasitoids endemic to sub-Saharan Africa (Daane *et al.*, 2011; Rugman-Jones *et al.*, 2009), but resulted in limited success (Borowiec *et al.*, 2012; Daane *et al.*, 2008; Daane *et al.*, 2015).

In addition to the sub-Saharan Africa species *P. lounsburyi* and *P. humilis*, *Psyttalia concolor* (Szépligeti), a koinobiont endoparasitoid of *B. oleae* found on wild and cultivated olives, is used in the Mediterranean region for augmentative control and propagative and inundative releases (Kapatos *et al.*, 1977; Liaropoulos *et al.*, 1977; Liotta & Mineo, 1968; Monastero & Delanoue, 1966; Raspi, 1995; Raspi & Loni, 1994; Silvestri, 1939). Morphologically, *P. concolor* is virtually indistinguishable from *P. humilis*, which has sometimes been treated as its junior synonym (Wharton & Gilstrap, 1983). *Psyttalia concolor* was first identified as a parasitoid of the olive fruit fly in Tunisia, and is also considered native to Sicily, southern Sardinia and southern Calabria (Caleca, *et al.*, 2017; Monastero, 1931; Silvestri, 1939; Szépligeti, 1910). More recently, *P. concolor* was found in various areas of coastal Tuscany (Raspi *et al.*, 2007). *Psyttalia concolor* has also been reared from medfly (*Ceratitis capitata* Wiedemann) infesting argan fruit (*Argania spinosa* L., Sapotaceae) in Morocco (Balachowsky & Mesnil, 1935; Debouzie & Mazih, 1999). *Psyttalia humilis* was described by Silvestri (1913), based on specimens reared from pears infested by medfly in Constantia (Cape Town, South Africa). The species has been reared from *B. oleae* collected from wild olives (*Olea europaea* L. subsp. *cuspidata*) (Wall ex G. Don Cif.) in Kenya and South Africa (Copeland *et al.*, 2004; Powell *et al.*, 2019), and may also be a parasitoid of *Bactrocera*

biguttula (Bezzi), another olive fruit fly infesting wild olives in South Africa, Namibia, and Kenya (Mkize *et al.*, 2008; Rugman-Jones *et al.*, 2009; Teixeira da Costa *et al.*, 2019).

Psyttalia lounsburyi was described by Silvestri (1913) as a parasitoid of the olive fruit flies infesting wild olives collected in South Africa, with *B.* oleae as the only confirmed host; however, the species could also be a parasitoid of *B. biguttula*. *Psyttalia humilis* was found in sub-Saharan Africa emerging from coffee beans (*Coffea arabica* L.) infested by *Ceratitis capitata*, and wild and cultivated olives infested by *B. oleae* and *B. biguttula*. Although very similar to *P. concolor*, these specimens were found to be genetically distinct from their Mediterranean counterpart, and were given the current designation of *P. humilis* (Rugman-Jones *et al.*, 2009).

Insect mitochondrial genomes are powerful sources of genetic information for the reconstruction of phylogenetic relationships due to their maternal inheritance, lack of recombination, conserved gene components and relatively small size (approximately 16, 000 bp) (Boore, 2006). The typical insect mitogenome is a circular molecule composed of 37 genes, of which 13 protein-coding genes, 22 transfer genes (tRNAs), two ribosomal genes (rRNAs) and an A+T rich region presumed to contain the origins of replication and transcription (Boore *et al.*, 1998). Insect mitogenomes have been rapidly accumulating in public databases due to their potential utility for resolving phylogenetic relationships (Arnason *et al*., 2002). Some particular features of mitogenomes have also been subject of current research, including substitutional rate heterogeneity, compositional bias and variation in genome organization (Castro *et al.*, 2002). This work reports the first mitochondrial genomes of *Psyttalia concolor, Psyttalia humilis* and *Psyttalia lounsburyi,* three Braconidae wasps known to parasitize the olive fruit fly, *B. oleae.* The three new mitochondrial sequences were characterized comparatively to other available braconid mitogenomes, and used to obtain further insights into the evolutionary relationships and gene rearrangements within Braconidae and particularly the Opiinae subfamily.

5.3 Materials and methods

5.3.1 Sample collection and morphological identification

Adult specimens of *Psyttalia humilis* and *P. lounsburyi* were reared from the fruits of wild olive trees (*O. europaea* L. subsp. *cuspidata*) collected in April and May 2016 at Grahamstown (33.3194962°S, 26.5170514°E) and Stellenbosch (33.9951346°S,

18.8675622°E), in the Western Cape province of South Africa. Adult specimens of *Psyttalia concolor* were reared from cultivated olive fruits (*O. europaea* L. subsp. *europaea* var. *europaea*) collected in November 2014 at Constância (39.4781°N, 8.3372°W) in the Ribatejo province of Portugal (Figure 5.1). Morphological species identification was performed on ethanol-preserved adults specimens using the taxonomic keys and photographic images available in the Parasitoids of Fruit infesting Tephritidae (PAROFFIT) database (http://paroffit.org) (Wharton and Yoder), and previous descriptions (Silvestri, 1913).

5.3.2 DNA extraction, PCR and sequencing

Total DNA was extracted from one adult specimen for each species using a standard SDS/Proteinase K method for *P. concolor* and a standard phenol-chloroform method for *Psyttalia humilis* and *P. lounsburyi*. The complete mitogenome of *P. concolor* (15,308 bp) was obtained by Sanger sequencing, after conventional PCR amplification in overlapping fragments. The mitogenome of *P. concolor* was recovered by a multi-step strategy starting with attempts to amplify "seed" regions with primers designed on the basis of the two braconid mitogenome sequences available at the time: *D. longicaudata* and C*. vestalis*. The seed regions thus obtained were then iteratively extended using a primer specific for *P. concolor* and one designed on the basis of the other braconid sequences. Once gaps between the regions were deemed to be bridgeable by PCR amplification, this was carried out using *P. concolor* specific primers. PCR amplification of the complete *P. concolor* mtDNA was achieved in eighteen segments, while shorter versions of four of these (marked with an asterisk) were also used to obtain sequence information (Table S12). Amplification reactions were carried out in a 25 µL mixture containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20 (Fermentas, Vilnius, Lithuania), 3.0 mM $MgCl_2$ (Fermentas), 0.5 mM of each deoxy-NTP (Fermentas), 25 pmol of each primer (Macrogen, Seoul, Republic of Korea) and 2.5 U of Taq DNA polymerase (Fermentas). Three different hot-start and touchdown cycling protocols were used: a) PAS1, consisting of 95°C for 5 min; 3 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 2 min; 3 cycles of 95°C for 30 s, 57°C for 1 min and 72°C for 2 min; 3 cycles of 95°C for 30 s, 54°C for 1 min and 72°C for 2 min; 38 cycles of 95°C for 30 s, 58°C for 1 min and 72°C for 2 min; and 72°C for 5 min; b) PAS2, consisting of 95°C for 5 min; 2 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 2 min; 2 cycles of 95°C for 30 s, 58°C for 1 min and 72°C for 2 min; 2 cycles of 95°C for 30 s, 56°C for 1 min and 72°C for 2 min; 38 cycles of 95°C for 30 s, 58°C for 1 min and 72°C for 2 min; and 72°C

for 5 min; c) PAS3, differing from PAS2 in that in the extension steps the temperature was decreased to 68°C and the time increased to 3 min. Following treatment with ExonucleaseI (Fermentas) and Shrimp Alkaline Phosphatase (Fermentas), PCR products were Sanger-sequenced by Macrogen Europe (Amsterdam, The Netherlands). Cycling protocols and primers used for amplification and sequencing of each segment are listed Table S12.

Psyttalia humilis and *P. lounsburyi* were sequenced using the Ion Torrent Proton™ sequencing platform (ThermoFisher Scientific, Waltham, MA, USA) at the Central Analytical Facilities of Stellenbosch University, South Africa. Sequence libraries were prepared using the NEXTflex™ DNA Sequencing Kit for Ion Platforms (PerkinElmer, Waltham, MA, USA) according to the BI00 Scientific v15.12 protocol. Libraries were pooled and sequenced using the Ion PI HiQ™ Sequencing Solutions Kit (Life Technologies, CA, USA).

Figure 5.1. Adult specimens representative of three *Psyttalia* species (Hymenoptera: Braconidae): (A) *Psyttalia concolor* male (scale bar = 1 mm); (B) *Psyttalia humilis* male (scale bar = 1 mm); (C) *Psyttalia lounsburyi* male (scale bar = 100 µm).

5.3.3 Mitogenome annotation and statistical analyses

The complete mitogenome of *P. concolor* was assembled using CLCBio Main Workbench v6.9, with some manual corrections. The NGS reads for *P. humilis* and *P. lounsburyi* were mapped to the complete *P. concolor* mitogenome sequence, and assembled using Geneious Prime v2019.1 (https://www.geneious.com). Open reading frame searches for the identification of protein-coding genes (PCGs) were performed using Geneious Prime, using the invertebrate mitochondrial translation table. Transfer RNAs (tRNAs) were predicted with the ARWEN software, using the default composite metazoan mitochondrial code (Laslett & Canbäck, 2008). Ribosomal RNAs were estimated by BLASTn search on NCBI (https://blast.ncbi.nlm.nih.gov). Overlapping regions and intergenic spacers were counted manually. Nucleotide composition and AT and GC asymmetries were calculated using Geneious Prime as AT-skew = (A-T)/(A+T); GC-skew = (G-C)/(G+C).

5.3.4 Phylogenetic analyses

Complete and partial mitogenomes of cyclostomes and non-cyclostomes available in Genbank were used, together with the three new mitogenomes described here, for the phylogenetic reconstruction of Braconidae, with *Diadegma semiclausum* and *Enicospilus* sp. (Ichneumonidae) as outgroups (Table S14). In line with Li *et al.*, 2016, analysis was restricted to PCGs, with the exception of ND2 (due to variable levels of completeness among the genomes) and to the first and second codon positions (due to the temporal depth of analysis). Sequences for each of the PCGs were aligned using Translator-X, poorly aligned regions were removed with G-blocks (with additional minor manual corrections) and the 24 partitions corresponding to the first and second codon positions were separated using MEGA7. Subsequent analysis were then performed using either these 24 partitions or the 15 partition subset used by (Li *et al.*, 2016). Three different partitioning schemes were tested for each of these sets, the one selected by PartitionFinder, partition by codon position alone or partition by codon position and strand. Phylogenetic analyses were implemented in the BEAST1.8.4 software package (Drummond *et al.*, 2012; Lemey *et al.*, 2009) available on the CIPRES Science Gateway V3.3 portal (www.phylo.org) with separate GTR + I + G (4 gamma categories) substitution models and lognormal relaxed clock models for each partition, but as a single global tree model. The tree was left unconstrained except for monophyly requirements for both Braconidae and Ichneumonidae. A Yule process tree prior was used, priors for Braconidae and Braconidae-Ichneumonidae divergence dates were based on recently published data (Branstetter *et al.*, 2018) and priors for mutation rates were

chosen on the basis of previous results for insects (Nardi *et al.*, 2010) and values obtained with jModelTest (Posada, 2003). Runs were performed for 10 (test runs) or 30 (final runs) million generations, with a 10% generation burn-in and sampling every 1000 generations. Trees were summarized and annotated using TreeAnnotator v1.8.1 (Drummond *et al.*, 2012), and drawn using FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

5.4 Results

5.4.1 Sequencing of *Psyttalia* mitogenomes

The mitochondrial genome of *Psyttalia concolor* using Sanger sequencing was found to be comprised of 15,308 bp. The Ion Torrent Proton™ run resulted in 34,002,499 reads (average read length of 177 bp) for *P. humilis* and 11,637,157 reads (average read length of 142 bp) for *P. lounsburyi*. The average coverage for the final sequence of *P. humilis* was 2,190.4x (186,659 mapped reads), and 80.7x for *P. lounsburyi* (7,181 mapped reads).

5.4.2 Mitochondrial organization and base composition

The mitogenomes of *P. concolor, P. humilis* and *P. lounsburyi* had the typical metazoan gene content (Table 5.1), with 13 PCGs, two rRNAs, and 22 tRNAs (Figure 5.2). The total sequence length of *P. concolor* (14,859 bp), *P. humilis* (14,861 bp) and *P. lounsburyi* (14,797 bp) was within the range found for other Braconidae (average excluding the putative control region = 12, 822 bp). Nine PCGs (*ND2, COI, COII, ATP8, ATP6, COIII, ND3, ND6, CYTB*) and 12 tRNAs ($tRNA^{Trp}$, $tRNA^{Leu1}$, $tRNA^{His}$, $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Ala}$, $tRNA^{Arg}$, $tRNA^{Asn}$, $tRNA^{Ser1}$, $tRNA^{Glu}$, $tRNA^{Thr}$, $tRNA^{Ser2}$) were found on the majority strand (J-strand), and the remaining four PCGs (*ND5, ND4, ND4L, ND1*), 10 tRNAs ($tRNA^{Gln}$, $tRNA^{Tyr}$, $tRNA^{Cys}$, $tRNA^{Asp}$, $tRNA^{Phe}$, $tRNA^{Pro}$, $tRNA^{Leu2}$, $tRNA^{Val}$, $tRNA^{Ile}$, $tRNA^{Met}$), and two rRNAs (lrRNA and srRNA) were found on the minority strand (N-strand). Gene order and direction were conserved among the three *Psyttalia*.

Strand asymmetry is generally reflected by AT-skew, as expressed by (A-T)/(A+T), and GC skew, (G-C)/(G+C) (Perna & Kocher, 1995). Positive AT skew values indicate more A than T on the target strand, and positive GC skew indicate more G than C, and vice versa. In insect mitochondrial genomes, the two DNA strands are referred to as the majority, or J, strand (light strand in mammal mitochondrial genomes), which encode more genes compared to the minority, or N, strand (heavy strand in mammal mitochondria genomes) (Simon *et al.*, 1994). The nucleotide composition of the *P. concolor, P. humilis* and *P.*

lounsburyi mitogenomes was highly biased towards A and T for the complete sequences (A+T = 84%, 83.9% and 83.4%, respectively) (Table S15). An AT-bias was also present in PCGs (average $_{Psyttalia}$ = -0.07), tRNAs (average $_{Psyttalia}$ = -0.01), and rRNAs (average $_{Psyttalia}$ = -0.07). The A+T content was higher for protein-coding genes encoded on the majority strand (average $_{Psyttalia}$ = 0.71) than for the minority strand (average $_{Psyttalia}$ = 0.10). The highest A+T content for *P. concolor* was found in *ND6* (89.9%), and in *ATP8* (90.4%) for both *P. humilis* and *P. lounsburyi.* The three mitogenomes had a negative AT-skew (average $_{Psyttalia}$ = -0.06) and a positive GC-skew (average $_{Psyttalia}$ = 0.19), similarly to the averages of the other 28 Braconidae (AT-skew = -0.04, CG-skew = 0.15) (Table S16). Codon usage contributed to the negative AT-skew in the three species, as these bases were overrepresented at the third codon position in all amino acids (Figure S11). The third codon position was also biased towards A and T when considering the J- and N-strands separately (Figure S12).

Table 5.1. Main features of the near-complete mitochondrial genomes of *Psyttalia concolor*, *Psyttalia humilis* and *Psyttalia lounsburyi*. AC – anticodon. IGN – intergenic (+) and overlapping nucleotides (-). J-strand – majority strand; N-strand – minority strand.

	Psyttalia concolor							*Psyttalia humilis*						*Psyttalia lounsburyi*					
Gene	**Location**	**Strand**	**Size**	**AC**	**Start**	**Stop**	**IGN**	**Location**	**Size**	**AC**	**Start**	**Stop**	**IGN**	**Location**	**Size**	**AC**	**Start**	**Stop**	**IGN**
COX1	1 - 1534	J	1534	-	ATG	T-	0	1 - 1539	1539	-	ATG	TAA	-6	1 -1539	1539	-	ATG	TAA	-6
tRNA^{Leu}	1535 - 1602	J	68	TAA	-	-	+8	1534 - 1601	68	TAA	-	-	+8	1534 -1602	69	TAA	-	-	+8
COX2	1611 - 2267	J	657	-	ATA	TAA	+163	1610 - 2266	657	-	ATA	TAA	+168	1611 - 2267	657	-	ATA	TAA	+168
tRNA^{Asp}	2431 - 2500	N	70	GTC	-	-	-1	2434 - 2503	70	GTA	-	-	-1	2435 - 2502	68	GTC	-	-	0
tRNA^{His}	2500 - 2567	J	68	GTG	-	-	-1	2503 - 2570	68	GTG	-	-	-1	2503 - 2568	66	CAC	-	-	+1
tRNA^{Lys}	2567 - 2637	J	71	TTT	-	-	0	2570 - 2640	71	TTT	-	-	0	2570 - 2640	71	TTT	-	-	0
ATP8	2638 - 2793	J	156	-	ATA	TAA	-22	2641 - 2796	156	-	ATA	TAA	-22	2641 - 2796	156	-	ATT	TAA	-22
ATP6	2772 - 3461	J	690	-	ATT	TAA	+9	2775 - 3464	690	-	ATT	TAA	+9	2774 - 3463	690	-	ATT	TAA	+3
COX3	3471 - 4259	J	789	-	ATG	TAA	0	3474 - 4262	789	-	ATG	TAA	0	3467 - 4255	789	-	ATG	TAA	0
tRNA^{Gly}	4260 - 4324	J	65	GGA	-	-	+14	4263 - 4323	61	TCC	-	-	+17	4256 - 4321	66	TCC	-	-	+14
ND3	4339 - 4723	J	385	-	ATT	T-	0	4341 - 4727	387	-	ATT	TAG	-3	4336 - 4713	378	-	ATT	TAG	-3
tRNA^{Ala}	4724 - 4785	J	62	TGC	-	-	-1	4725 - 4787	63	TGC	-	-	-1	4711 - 4773	63	TGC	-	-	-1
tRNA^{Arg}	4785 - 4851	J	67	TCG	-	-	-7	4787 - 4853	67	ACG	-	-	-7	4773 - 4839	67	ACG	-	-	-7
tRNA^{Asn}	4845 - 4911	J	67	GTT	-	-	-3	4847 - 4913	67	AAC	-	-	-3	4833 - 4899	67	GTT	-	-	-3
tRNA^{Ser}	4909 - 4975	J	67	AGA	-	-	-1	4911 - 4977	67	AGA	-	-	-1	4897 - 4963	67	AGA	-	-	-1
tRNA^{Glu}	4975 - 5039	J	65	TTC	-	-	-2	4977 - 5041	65	TTC	-	-	-2	4963 - 5027	65	GAA	-	-	-2
tRNA^{Phe}	5038 - 5101	N	64	GAA	-	-	0	5040 - 5104	65	GAA	-	-	-27	5026 - 5090	65	GAA	-	-	-27
ND5	5102 - 6761	N	1660	-	ATA	T-	+26	5078 - 6763	1686	-	ATA	TAA	+26	5064 - 6749	1686	-	ATA	TAA	+23
ND4	6788 - 8110	N	1323	-	ATG	TAA	-7	6790 - 8112	1323	-	ATG	TAA	-7	6773 - 8098	1326	-	ATG	TAA	-7
ND4L	8104 - 8400	N	297	-	ATT	TAA	+7	8106 - 8402	297	-	ATT	TAA	+7	8092 - 8388	297	-	ATT	TAA	+8
tRNA^{Pro}	8408 - 8474	N	67	TGG	-	-	0	8410 - 8476	67	TGG	-	-	0	8397 - 8460	64	TGG	-	-	+5
tRNA^{Thr}	8475 - 8538	J	64	TGT	-	-	+11	8477 - 8540	64	TGT	-	-	+11	8466 - 8527	62	TGT	-	-	+12
ND6	8550 - 9115	J	566	-	ATG	TA-	0	8552 - 9118	567	-	ATG	TAA	-1	8540 - 9106	567	-	ATG	TAA	+2
CYTB	9116 - 10244	J	1129	-	ATG	T-	0	9118 - 10248	1131	-	ATG	TAA	-2	9109 - 10239	1131	-	ATG	TAA	-2
tRNA^{Ser}	10245 - 10311	J	67	TGA	-	-	0	10247 - 10313	67	TGA	-	-	-2	10238 - 10305	68	TGA	-	-	-2
ND1	10312 - 11269	N	958	-	ATT	T-	0	10312 - 11271	960	-	ATT	TAA	+4	10304 - 11263	960	-	ATT	TAA	0
tRNA^{Leu}	11270 - 11335	N	66	TAG	-	-	0	11276 - 11337	62	TAG	-	-	0	11264 - 11330	67	TAG	-	-	0
lrRNA	11336 - 12623	N	1288	-	-	-	0	11338 - 12626	1289	-	-	-	0	11331 - 12608	1278	-	-	-	-1
tRNA^{Val}	12624 - 12689	N	66	GTA	-	-	0	12627 - 12692	66	GTA	-	-	0	12608 - 12672	65	TAC	-	-	1
srRNA	12690 - 13436	N	747	-	-	-	0	12693 - 13437	745	-	-	-	0	12674 - 13427	754	-	-	-	0
tRNA^{Ile}	13437 - 13500	N	64	GAT	-	-	+2	13438 - 13501	64	GAT	-	-	+3	13428 - 13491	64	ATC	-	-	+4
tRNA^{Met}	13503 - 13568	N	66	CAT	-	-	0	13505 - 13570	66	CAT	-	-	0	13496 - 13562	67	CAT	-	-	0
A+T-rich region	13569 - 14017	-	449	-	-	-	0	13571 - 14021	451	-	-	-	0	13563 - 13748	185	-	-	-	0
tRNA^{Gln}	14018 - 14091	N	74	TTG	-	-	+3	14022 - 14093	72	TTG	-	-	+3	13749 -13764	15	TTG	-	-	+3
ND2	14095 - 15115	J	1021	-	ATA	T-	0	14097 - 15119	1023	-	ATA	TAA	-3	13768 - 14790	1023	-	ATA	TAA	-3
tRNA^{Trp}	15116 - 15183	J	68	TCA	-	-	-4	15117 - 15185	69	TCA	-	-	-4	14788 - 14856	69	TCA	-	-	-3
tRNA^{Tyr}	15180 - 15245	N	66	GTA	-	-	-1	15182 - 15247	66	GTA	-	-	-1	14854 - 14917	64	GTA	-	-	+1
tRNA^{Cys}	15245 - 15308	N	64	GCA	-	-	0	15247 - 15311	64	GCA	-	-	0	14919 - 14982	64	GCA	-	-	0

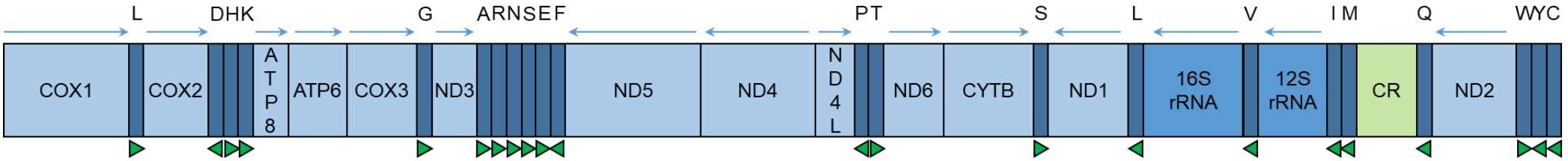

Figure 5.2. Linear map of the partial mitochondrial genome of *Psyttalia* species, drawn to scale. Arrows indicate the direction of gene transcription. tRNA genes are designated by single-letter abbreviations.

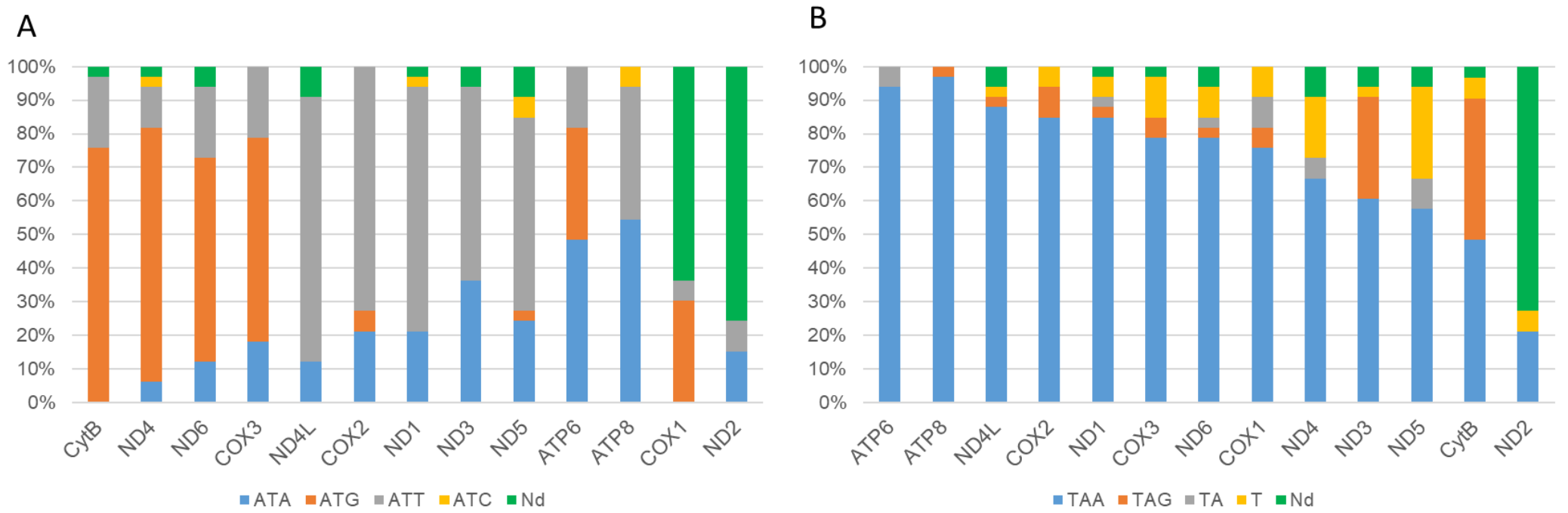

Figure 5.3. Usage of (A) start codons and (B) stop codons in the complete set of protein-coding genes in 31 partial and complete mitochondrial sequences of Braconidae species.

5.4.3 Protein-coding genes

The 13 PCGs made up an average of 11, 190 bp (24,5%) of the near-complete mitogenomes of each *Psyttalia* species. The most frequently used start codon was ATT, which was conserved in all PCGs of *Psyttalia* (Table S15.; Figure 5.3.). The start codon ATC was only present in five species including *Diadegma semiclausum, Sigalphus bicolor, Acanthormius* sp*., Elasmosoma* sp*.,* and *Macrocentrus camphoraphilus* in four PCGs (*ATP8, ND1, ND4* and *ND5*). The start codon of nine PCGs in 26 Braconidae was not determined presumably due to incomplete sequencing. The stop codon was also not determined in nine PCGs in 25 Braconidae sequences. Among Braconidae, the most frequently used stop codon was TAA, and incomplete stop codons (TA- and T-), putatively completed by posttranscriptional polyadenylation (Bratic *et al.*, 2016), were found in 12 PCGs.

5.4.4 Transfer RNAs

The typical 22 tRNAs were found in the three *Psyttalia* species. The tRNAs were organized into two main clusters: A-R-N-S-E-F and W-Y-C. All tRNAs were predicted to fold into the typical cloverleaf structure except for $tRNA^{Ser}$, for which the dihydrouridine (DHU) arm was reduced to a simple loop, a frequent occurrence in metazoans (Jühling *et al.*, 2012). Fourteen non-canonical G-U pairs and two U-U pairs were found in ten of the predicted tRNA structures of *P. concolor*. Fifteen non-canonical G-U pairs and two U-U pairs were found in ten of the predicted tRNA structures of *P. humilis.* Fourteen non-canonical G-U pairs and three U-U pairs were found in eleven of the predicted tRNA structures of *P. lounsburyi.* The highest number of non-canonical pairs (20) was found in *Mirax* sp., which was above the overall average (excluding *Mirax* sp.) of 12 mismatches. Non-canonical pairs differed across all 31 partial and complete Braconidae mitochondrial sequences with the lowest mismatches observed in $tRNA^{Trp}$ (3) and the highest mismatches observed in $tRNA^{Gly}$ (46), and were more frequent in the DHU and AA arms.

5.4.5 Non-coding, intergenic and overlapping regions

The AT-rich region putatively containing the control region was not successfully recovered for any of the three *Psyttalia* species, most probably due to the extremely high A+T content, as commonly is the case in attempts to sequence complete hymenopteran mitochondrial genomes (Cameron *et al.*, 2008; Castro & Dowton, 2005; Castro *et al.*, 2006). A+T-rich regions were only found to be annotated in four Braconidae (14, 3 %) (*C. vestalis, D.*

longicaudata, M. camphoraphilus and *S. agrilis,* average length = 529 bp) Among Braconidae, the intergenic space between *COX2* and $tRNA^{Asp}$ was the longest in *P. concolor* (163 bp), *P. humilis* and *P. lounsburyi* (168 bp).Gene overlapping was found at 6, 8 and 7 locations for *P. concolor, P. humilis* and *P. lounsburyi* respectively, with the longest between *ATP6* and *ATP8* (*P. concolor,* 22 bp) and between $tRNA^{Phe}$ and *ND5* (*P. humilis* and *P. lounsburyi,* 27 bp).

5.4.6 Gene rearrangements in Braconidae

Gene rearrangements can be classified into three classes: translocation, which involves gene movement across a protein-coding gene, local inversion involving genes that "jump" from one strand to the other but remain in the same position i.e. from the majority strand to the minority strand and vice versa, and shuffling with remote inversion which refers to translocation and inversion (Dowton *et al*., 2003; Wei *et al.*, 2010). Several gene rearrangements were found between *P. concolor, P. humilis* and *P. lounsburyi* relative to the other cyclostome braconid wasps, and all rearrangements involved tRNA genes. The ancestral organization of the *COX2* and *ATP8* junction is inferred as *COX2*-K-D-*ATP8;* however, all three *Psyttalia* species and seven other cyclostome braconids had the derived state of *COX2*-<u>*D*</u>-H-K-*ATP8,* in which <u>*D*</u> is inverted (i.e. encoded on the N-strand rather than the J-strand), and H was inverted and translocated from its original location between *ND5* and *ND4* to its new location between D and K (Figure 5.4). Additionally, a different derived state of *COX2*-<u>*D*</u>-K-H-*ATP8* was observed in *Pambolus* sp. with <u>*D*</u> and H genes inverted, and K shuffled to its new location between inverted genes <u>*D*</u> and H. Furthermore, *COX2*-D-K-*ATP8* was observed in *Histeromerus* sp. in which K shuffled positions with the newly inverted D gene.

The ancestral I-Q-M cluster was proposed to be situated between the AT-rich region and *ND2*. All *Psyttalia* species had the derived state of <u>*I*</u>-<u>*M*</u>-AT-rich region-Q where <u>*I*</u> and <u>*M*</u> were inverted and translocated from their ancestral positions to their new positions between srRNA and the the AT-rich region. The same derived state of the <u>*I*</u>-<u>*M*</u>-AT-rich region-Q cluster was observed in *S. agrili*, *D. longicaudata* and *A. gifuensis*; however, Q and I was not annotated for *D. longicaudata* and *A. gifuensis,* respectively. Furthermore, the AT-rich region was not determined in *A. gifuensis*, possibly due to the incomplete sequencing.

The ancestral W-C-Y cluster was proposed to be situated between *ND2* and *COX1*. *Psyttalia concolor, P. humilis* and *P. lounsburyi* had identical derived states of W-Y-C in which C and

Y have shuffled (i.e. swapped positions). *Spathius agrili* had the derived state of C-W-Y in which W and C have shuffled.

Furthermore, the *Psyttalia* species had unique gene rearrangements: 1) the ancestral C and Y from the W-C-Y cluster are located between *ND2* and *COX1*; however, in all *Psyttalia* species, C and Y were found to *i.e.* the tRNA genes were found to be on the same strand but in a different position compared to the ancestral arrangement; 2) the ancestral P and T are situated between *ND4L* and *ND6*; however, in all *Psyttalia* species, P and T were found to be shuffled.

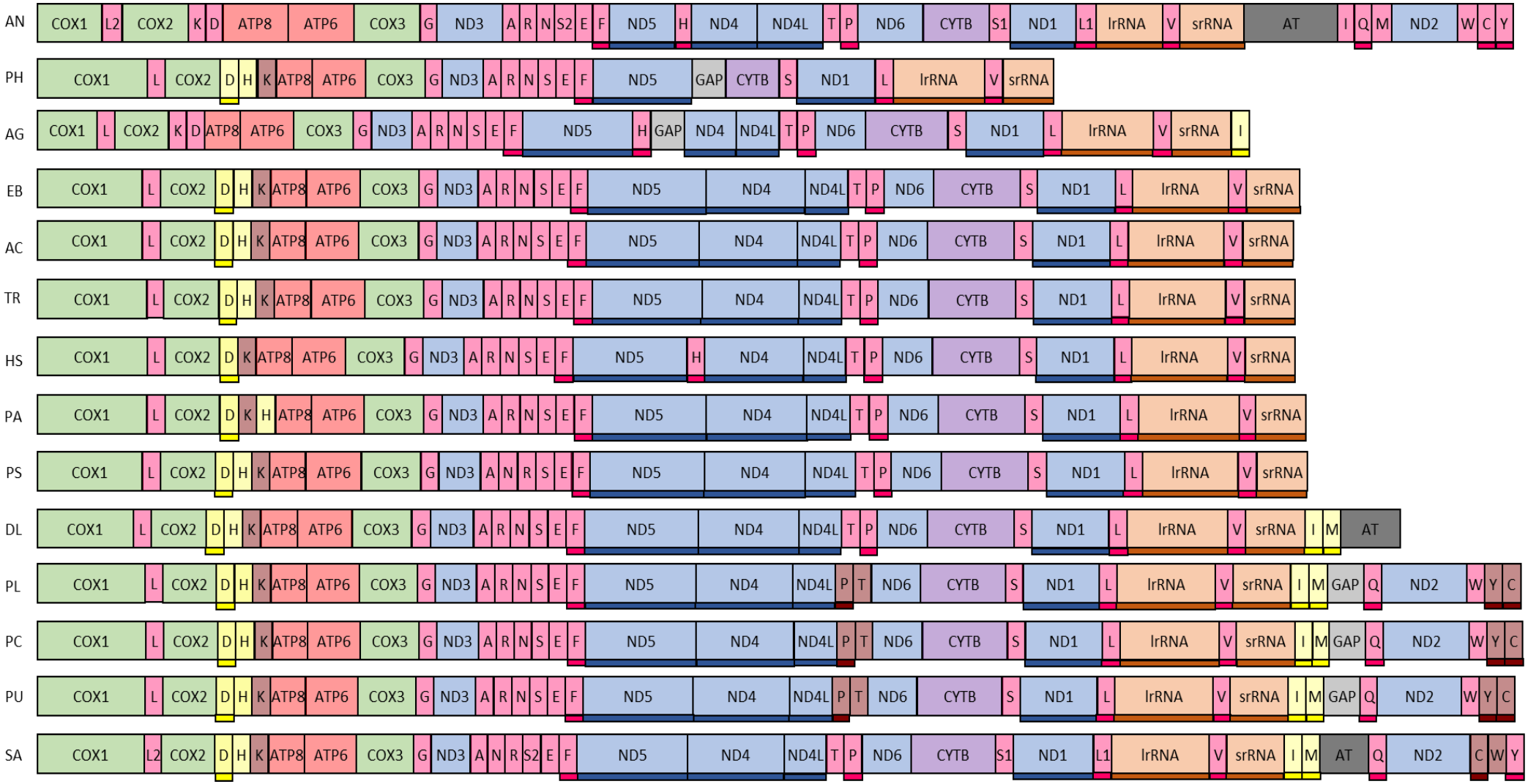

Figure 5.4. Gene arrangements in the mitochondrial genomes of 13 species of cyclostome wasps (Hymenoptera: Braconidae). Non-underscored boxes represent genes in majority strand and underscored boxes represent genes in the minority strand. Yellow – gene was inverted or remotely inverted; Brown – gene was translocated or shuffled. AT – A+T-rich region; AN – ancestral arrangement. PH – *Phaenocarpa* sp., AG – *Aphidius gifuensis*, EB – *Euurobracon breviterebrae*, AC – *Acanthormius* sp., TR – *Triraphis* sp., HS – *Histeromerus* sp., PA – *Pambolus* sp., PS – *Pseduognapton* sp., DL – *Diachasmimorpha longicaudata*, PL – *Psyttalia lounsburyi,* PC – *Psyttalia concolor*, PU – *Psyttalia humilis*, and SA – *Spathius agrili*.

5.4.7 Phylogenetic reconstruction of *Psyttalia*

Our main phylogenetic analysis was conducted on the same set of 15 genes and codon position partitions used by (Li *et al.*, 2016) and yielded the tree depicted in Figure 5.5, with the three *Psyttalia* species clustering with *D. longicaudata*, as expected. *Psyttalia concolor* and *P. humilis* were recovered as sister species, having diverged approximately 2.5 MYA, while *P. lounsburyi* occupies the most basal position among the three *Psyttalia*, having diverged from the two about 12 MYA. Li *et al.*, 2016 reported topological variations depending on the data matrices and analytical methods used in analyses including all Braconid species. Therefore, such variations could also affect the positions of the *Psyttalia* species, and additional analysis were conducted using a data matrix containing 24 gene and codon partitions and/or different partition-clustering schemes. Several topological alterations were indeed observed when using these alternative analyses, but none of them involved any of the Opiinae species. Surprisingly, the monophyly of the cyclostomes was found to be sensitive to partition-clustering (but not the data matrix chosen), as *A. gifuensis* occupied the most basal position among all Braconids in the trees obtained with both alternative partition-clustering schemes.

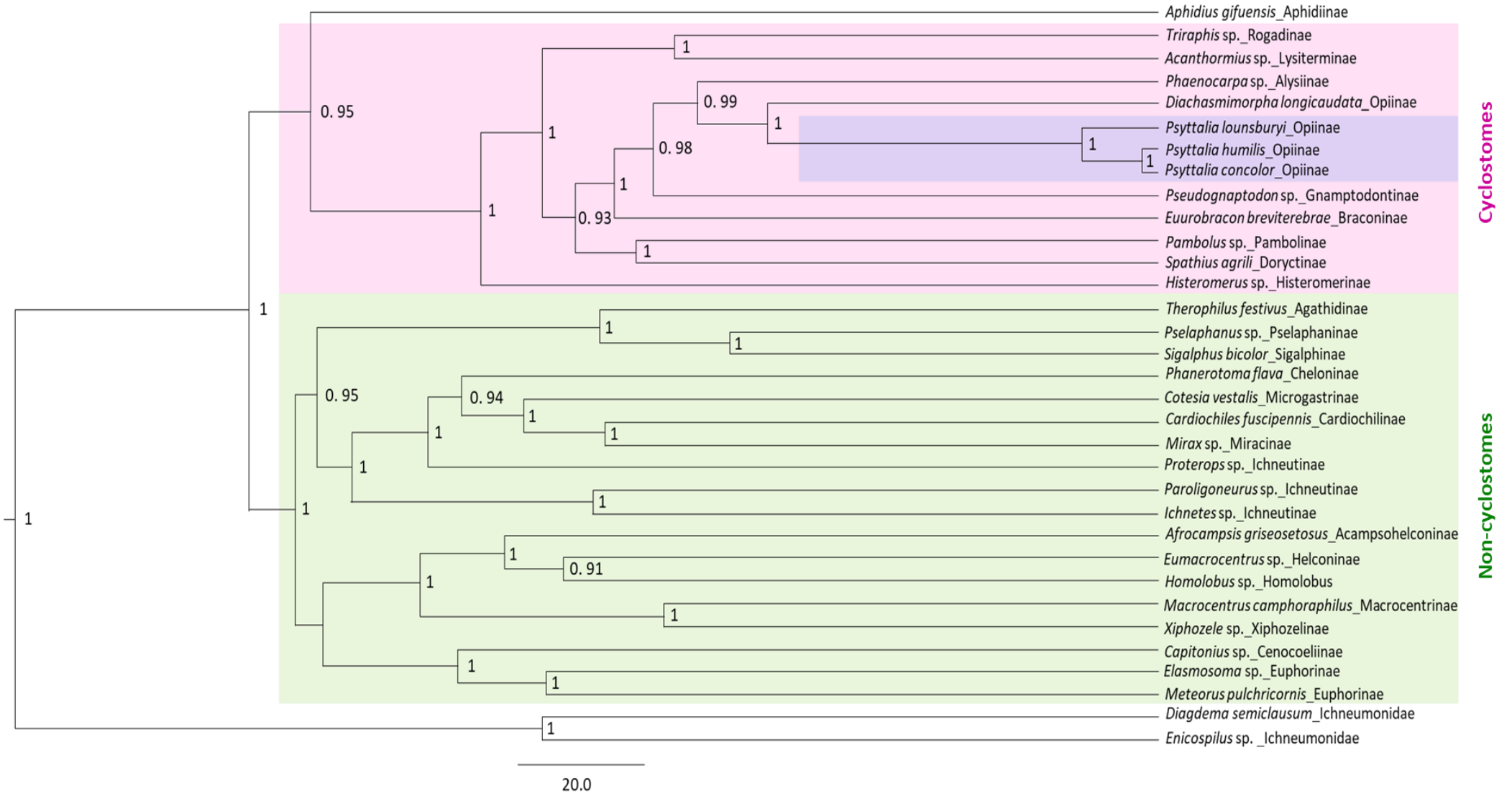

Figure 5.5. Phylogenetic relationships among 31 Braconidae species based on the same set of 15 genes and codon position partitions used by Li *et al.* 2016. Species survyed in this study are shaded in blue. Cyclostome braconids are shaded in pink and non-cyclostome braconids shaded in green. *Diadegma semiclausum* and *Enicosphilus* sp. (Ichneumonidae) used as outgroups. Values indicate Bayesian posterior probabilities. Scale bar repsrents the percentage of sequence divergence.

5.5 Discussion

5.5.1 Sequencing of *Psyttalia* mitogenomes

The advent of next-generation sequencing has allowed for the generation of a large number of sequences within a short period of time when compared to conventional Sanger sequencing (Chilana *et al.*, 2012). Next-generation sequencing has been applied for the rapid generation of sequence data, and more recently, for the assembly of complete mitochondrial sequences (Knaus *et al.*, 2011). Total genomic DNA extractions can contain high concentrations of mitochondrial DNA, which may then be overrepresented in the NGS data. This allows for both mining of mitochondrial genomes from the NGS data (Miller *et al.*, 2013), as well as its use to complete and correct published mitochondrial DNA sequences (Teixeira da Costa *et al.*, 2019). However, such strategies are not without its pitfalls, an extreme example of which is represented by the recovery of a complete mitochondrial genome of a rodent from an NGS-based genome sequence of a bat (Shi *et al.*, 2016). Furthermore, correct assembly of mitogenome NGS sequence data can be greatly hampered by the lack a good reference sequence, particularly in groups, such as the Hymenoptera, where alterations of gene order are common. We therefore started by obtaining the mitogenome of *Psyttalia concolor* by a conventional Sanger sequence-based strategy, which provided us with a reference sequence on which to assemble the NGS data from two congeneric species: *P. humilis* and *P. lounsburyi.*

5.5.2 General description of mitogenomes

Strand compositional bias is frequent in insect mtDNA and is presumed to be a result of the either the J- or N-strand in a single-stranded state, which exposes the strands to more DNA damage and the chance for repair during transcription and replication processes. This means that the exposed single-stranded DNA has a greater probability of deamination of C and A nucleotides resulting in greater frequencies of C and A content on the complementary strand (Francino & Ochman, 1997; Wei *et al.*, 2010). Usually, A+T content is higher than G+C on the majority strand; however, in some arthropods (Cameron *et al.*, 2008; Hassanin, 2006; Hassanin *et al.*, 2005; Kilpert & Podsiadlowski, 2006; Masta *et al.*, 2009), flatworms (Min & Hickey, 2007), brachiopods (Helfenbein *et al.*, 2001), echinoderms (Scouras & Smith, 2006) and fish (Wang *et al.*, 2007), strand asymmetry is reversed with less A than T and less C than G on the majority strand (Wei *et al.*, 2010). The *Psyttalia* species under

investigation exhibited strand asymmetry as the negative AT-skew values indicate less A than T and less C than G on the majority strand (Table S16).

Most protein-coding genes used ATT start codons and TAA stop codons or truncated TA- or T- stop codons. Previously, *ND1* has been found to use TTG as a start codon in some Hymenoptera, Coleoptera and Lepidoptera therefore reducing intergenic spacing and circumventing overlap with adjacent genes (Bae *et al.*, 2004; Sheffield *et al.*, 2008; Wei *et al.*, 2010). However, in the present study, TTG was not discovered as a start codon for *ND1* similarly to a previous study (Wei *et al.*, 2010). Majority of tRNAs display the cloverleaf structure and anticodons commonly used in insects. All *Psyttalia* species $tRNA^{Lys}$ use TTT as anticodons rather than the normal CTT. This finding is similar to results found in an earlier study in which $tRNA^{Lys}$ used anticodon TTT (Wei *et al.*, 2010). The usage of irregular anticodons in this tRNA seems to be associated with gene rearrangement (Wei *et al.*, 2009). The region around the *ND2* gene and A+T-rich region mitochondrial sequences was not sequenced for most of the Braconidae. High A+T content, unexpected gene rearrangement and stable stem-and-loop structures could have hampered sequencing, a common issue in the sequencing of hymenopteran mitochondrial genomes (Cameron *et al.*, 2008; Castro & Dowton, 2005; Castro *et al.*, 2006).

5.5.3 Gene rearrangements in Braconidae

Comparative mitogenomics of closely related taxa have indicated gene rearrangements are infrequent with a low likelihood of convergence owing to the large number of different possible combinations (Boore *et al.*, 1995; Moritz *et al.*, 1987). As such, mitochondrial gene rearrangements can be particularly useful resolving ancient evolutionary relationships (Boore *et al.*, 1995; Rokas & Holland, 2000). However, rearrangements are more common in some lineages, especially in arthropods, including Myriapoda (Negrisolo *et al.*, 2004), Hymenoptera (Dowton *et al.*, 1998), Hemiptera (Shao & Barker, 2003; Shao *et al.*, 2001; Shao *et al.*, 2001), Acari (Navajas *et al.*, 2000; Shao *et al.*, Araneae (Masta & Boore, 2004; Qiu *et al.*, 2005), and Isopoda (Kilpert & Podsiadlowski, 2006; Podsiadlowski & Bartolomaeus, 2006). Therefore, these lineages can be adequate models for studying the mechanisms underlying gene rearrangements (Wei *et al.*, 2010).

The *D*-H-K or *H*-D-K, arrangement has previously been found in many subfamilies of Braconidae, and it has been reported that both the inversion of D and the remote inversion of H were independent evolutionary events in this family (Dowton & Austin, 1998; Dowton

et al., 2002). Furthermore, an earlier study reported evidence of gene rearrangement in *ND3–ND5* junction in Hymenoptera, and that translocation of tRNA genes out of the *ND3–ND5* junction in which the A-R-N-S2-E-*F* cluster is present, has occurred more frequently than translocation of tRNAs into this junction (Dowton *et al.*, 2003). Mechanisms proposed to be responsible for gene rearrangements in hymenopteran mitochondrial genomes has gained interest (Dowton & Austin, 1998; Dowton *et al.*, 2002). Inter/intro- mitochondrial genome combination is alleged to be the most probable explanation for local inversions; however, we found no evidence of locally inverted genes in the 13 cyclostome mitochondrial sequences analysed in the present study. A “tandem duplication-random loss” model was developed to explain various gene rearrangements in vertebrate mitogenomes (Macey *et al.*, 1997; Moritz *et al.*, 1987), in which a segment of the DNA is duplicated through replication slippage or inaccurate termination of replication. Replication slippage involves denaturation and displacement of the DNA strands, resulting in mispairing of the complementary bases. Accurate replication of a circular molecule involves the termination point corresponding exactly to the replication origin. If an error occurs causing the termination point to precede the origin, a deletion occurs (Boore, 2000). Replication slippage or inaccurate termination of replication results in the mitochondrial genome being partially duplicated, i.e. containing duplicated copies of some genes and single copies of other genes. Novel gene rearrangement results from random deletion of one of each of the duplicated copies produced by tandem duplication (Boore, 2000). The build-up of random mutations determines which copy is lost, by disturbing normal gene function and producing a pseudogene that is further selected against and ultimately lost from the genome (San Mauro *et al.*, 2006).

Mitochondrial gene rearrangements in invertebrates seem to be distinct from vertebrates, as the duplication-random loss model cannot account for rearrangements involving inverted genes (Dowton *et al.*, 2003). However, shuffling is presumed to be the result of duplication/random loss. Furthermore, the tRNA clusters W-C-Y and D-H-K are frequently found to be shuffled; however, D was not only shuffled but also inverted which suggests that this may have resulted from two independent events due to separate mechanisms (Wei *et al.*, 2010). The gene arrangement of *Psyttalia* differed from all other available cyclostome mitogenomes in that two novel tRNA gene arrangements were found, C and Y, and P and T that were shuffled, whilst the remaining 10 cyclostomes had the ancestral arrangement of these tRNAs. A frequent gene arrangement event in braconid mitochondrial genomes is remote inversion of $tRNA^{His}$, $tRNA^{Ile}$ and $tRNA^{Met}$ but remote inversion could not be inferred

in previous studies due to incompleteness of sequence data (Dowton & Austin, 1998; Dowton *et al.*, 2003). However, remote inversions may be a result of two separate recombination events (Wei *et al.*, 2010).

5.5.4 Phylogenetic reconstruction of Braconidae

Phylogenetic relationships between braconid subfamilies have been a point of contention for many years (van Achterberg, 1976, 1984, 1993; Fischer, 1972; Quicke & Achterberg, 1990; Tobias, 1967) and only a few firm conclusions have been reached such as the general acceptation of two major groupings of subfamilies: cyclostomes and non-cyclostomes (van Achterberg, 1984; Askew & Shaw, 1986; Gauld, 1988). An early study provided a comprehensive phylogenetic tree based on morphological characters of the family Braconidae (Quicke & Achterberg, 1990). However, this study was criticized by several authors as morphology-based phylogenies are often subject to problems related to reductional synapomorphies as it is challenging to determine whether lost structures are a result of homologous or convergent events (van Achterberg, 1988; Gibson, 1985; Wharton *et al.*, 1992). Consequently, molecular data has been useful in describing subfamily relationships within the family or relationships within subfamilies (Belshaw & Quicke, 1997; Chen *et al.*, 2003; Gimeno *et al.*, 1997; Li *et al.*, 2003; Whitfield *et al.*, 2002).

Our phylogenetic analysis recovered the well-supported major lineages within the family Braconidae, such as the cyclostomes and non-cyclostomes (Figure 5.5). The three *Psyttalia* (Opiinae) species clustered with *Diachasmimorpha longicaudata* (Opiinae), as expected. Furthermore, *P. concolor* and *P. humilis* were recovered as sister species, while *P. lounsburyi* was found to be in the most basal position of the three *Psyttalia* species. Although *P. concolor* and *P. humilis* are morphologically indistinguishable, our analyses support the fact that the two species are genetically distinct from each other. One of the most widely studied groups within Braconidae is the subfamily Aphidiinae, which has long been thought as a distinct subfamily Aphidiidae. Earlier morphology-based phylogenies placed Aphidiinae as a sister clade to the non-cyclostome group (van Achterberg & Quicke, 1992; Quicke & Achterberg, 1990; Wharton *et al.*, 1992). However, as in many recent phylogenetic studies, our analyses recovered Aphidiinae as a sister group to the cyclostomes. For example, a study using nuclear 16S and 28S rDNA gene fragments along with a suite of morphological characters from 74 in-group taxa reported Aphidiinae as a sister group to the cyclostome braconids (Dowton *et al.*, 2002). A previous study compared seven mitochondrial genomes from seven subfamilies of Braconidae to assess the phylogenetic utility of mitochondrial

genomes found the subfamily Aphidiinae as a sister group to the cyclostomes (Wei *et al.*, 2010). Another study evaluating the phylogenetic relationships among 95 genera jointly representing 17 of the 18 currently recognized cyclostome braconids using mitochondrial *COI* and nuclear 28S rDNA gene fragments, in addition to morphological data, also placed the subfamily Aphidiinae, as well as subfamily Mesostoinae, as sister groups to the cyclostomes (Zaldivar-riverón *et al.*, 2006). Our phylogenetic analysis confirms the placement of subfamily Aphidiinae as a sister group to the cyclostomes. In addition, nucleotide sequences performed better compared to amino acid sequences in analyses of groups within Braconidae, supporting the idea that amino acid sequences are better suited for deeper-level relationships while nucleotide sequences are better for shallow-level relationships within Braconidae (Li *et al.*, 2003).

5.6 Conclusion

In this study, we reported three near-complete mitochondrial genomes of *Psyttalia concolor, Psyttalia humilis* and *Psyttalia lounsburyi* within the family Braconidae. The mitochondrial genomes of *Psyttalia* exhibited reversal of strand asymmetry, which is consistent with other Braconidae. Mitochondrial gene rearrangement varied substantially between the cyclostome mitochondrial genomes analysed, with novel gene rearrangements found in the *Psyttalia* species. Furthermore, remote inversion was common in cyclostome Braconidae. The variation in gene rearrangement among Braconidae suggests that gene rearrangement may be more diverse than previously reported. In addition, phylogenetic reconstruction supported the separation of the cyclostomes and non-cyclostomes, two major lineage groups identified in the family Braconidae. Our analyses also confirmed the subfamily Aphidiinae as a sister group to the cyclostome clade. As seen in recent studies including the present study, mitochondrial genomes have potential for inferring relationships at the subfamily, family and ordinal levels.

Chapter 6: Conclusion

The olive crop is historically, socially, and economically important worldwide. Not only are olives used as a food source, but it is also used in pharmaceuticals, cosmetics, and as traditional ointments in some religions and other rituals. Additionally, the added health benefit of consuming olive oil and table olives make it an attractive alternative to other animal and vegetable fats. Since olives form an essential component of the Mediterranean diet, it is understandable that the production of olive oil and table olives contribute significantly to the economy of the region, especially in leading olive-producing countries such as Spain, Italy, Portugal, and Greece. Recent years have seen a growth in the olive production in new areas such as Argentina, Australia, Chile, China, and the United States, and production is set to increase steadily. However, the growing olive-industry faces an agricultural pest, the olive fruit fly, with slow progress being made in its control and management. Effective control strategies are necessary, especially where this pest is most damaging, as the olive fruit fly has the ability to rapidly colonize new areas, thus posing a threat to virtually every olive-producing region.

Since commercial olive production and processing is relatively new in South Africa, little is known about native insect pests affecting cultivated olives, and their natural enemies. In fact, the non-threating status of the olive fruit fly in South Africa is hypothesized to be the result of native natural enemies maintaining fly populations at low levels. Moreover, South African olive production losses are mainly a result of olive seed wasps (OSW), presently not known to occur in other olive-producing regions, such as the Mediterranean Basin or California. Therefore, knowledge regarding the insects associated with olive fruit is relevant for the discovery and verification of OSW, as the lifestyles of several species is undetermined. Furthermore, since OSW are not known to occur in other olive-producing regions, there is no prior information that could inform the South African olive industry. The focus of this study was to comprehensively catalogue parasitoid and olive seed wasp species associated with wild and cultivated olives in the Western Cape province of South Africa, to identify and elucidate lifestyles of several putative seed wasp species, and to gain further insights into the phylogenetic relationships among Braconidae.

The survey of wild and cultivated olives in the Western Cape confirmed a higher degree of insect diversity in wild olives compared to cultivated olives, as well as a greater species diversity in the Western Cape compared to Eastern Cape province. Moreover, this survey

constitutes as the first comprehensive survey of insects associated with olive fruit in the Western Cape in the molecular era, since entomological surveys in the region were conducted in the early 1900s (Neuenschwander, 1982; Silvestri, 1913, 1914, 1915). More recently, the Eastern Cape province was surveyed but the study did not generate genetic data. In addition, this study contributed with the first barcoding sequences for several species including *Bracon celer*, *Utetes africanus*, *Eupelmus spermophilus*, *Eurytoma oleae*, *Eurytoma varicolor*, and *Sycophila aethiopica*.

In addition to cataloguing insects associated with olive fruit in the Western Cape province, a DNA-based species identification tool was developed to firstly, identify immature insect specimens; and secondly, to elucidate the lifestyles of putative OSW species. The development of this DNA-based tool is specifically crucial for industry purposes in that immature life stages of OSW can now be detected by screening olive seeds of wild and cultivated fruit. This will allow for early detection of OSW and assist in the development of control measures. In addition to the development of a DNA-based tool, the lifestyles of two species, *Eurytoma oleae* and *Sycophila aethiopica* were identified as OSW, a subject of speculation for over a century. This identification tool proves to be a quick and cost-effective method of detecting the presence and identifying the species of OSW.

In light of the olive fruit fly pest status in major olive-producing countries and the search for suitable biological control agents, it is imperative to study the *Psyttalia* species, especially the sub-Saharan *P. humilis* and *P. lounsburyi*, as they hold the potential of being utilized in biocontrol programs in areas where olive fruit fly infestation is high. The comparative analysis of the mitochondrial genomes of the three olive fruit fly parasitoids (*P. concolor, P. humilis* and *P. lounsburyi)*, revealed extensive rearrangement of tRNA genes, which seems to be a common occurrence within the family Braconidae. Previous authors observed that all mitochondrial gene rearrangements in Hymenoptera occurred after a parasitic lifestyle was adopted, yet the reason behind these gene rearrangements remains unclear. As gene rearrangements seem to be a common occurrence within Braconidae, future studies should invest in utilizing gene rearrangements as a phylogenetic tool for reconstructing phylogenies among Braconidae species since many of the relationships within this group are taxonomically contentious. Moreover, this study has generated the first mitochondrial genomes for the *Psyttalia* species.

Earlier studies reporting on the limited success of *P. humilis* and *P. lounsburyi* as biocontrol agents in California attributed the failure to four potential factors. Firstly, the limited numbers

of parasitoids available for release and rearing conditions used to produce more parasitoids. Optimal release strategies ideally involve large release of parasitoids at sites highly infested with *B. oleae.* Secondly, the parasitoids tolerance to extreme climatic conditions may have contributed to limited success in the establishment of parasitoids, as *P. lounsburyi* was previously reported (under laboratory conditions) to be better suited in parasitizing *B. oleae* (relative to *P. humilis*), as this species seems to be more cold tolerant compared to *P. humilis*. Thirdly, since *B. oleae* is also a specialist, insufficient host material during olive off-season intervals may hinder parasitoid establishment. This is because both *P. humilis* and *P. lounsburyi* do not enter a winter diapause; therefore, inadequate host availability presents a challenge for the survival of parasitoids emerging in early spring. Furthermore, in the native range of *P. humilis* and *P. lounsburyi,* wild olive trees fruit throughout the year thus providing a natural host reservoir continuously. As a result, future studies in biological control of *B. oleae* should take into account the parasitoid species' inherent ability to survive climatic extremes and poor host availability in the field. More importantly, since the domestication of the olive resulted in larger and fleshier fruit compared to wild olives and *B. oleae* is hypothesized to have coevolved with the olive, it is likely that this changed the parasitoid-host dynamics whereby parasitoid species with shorter ovipositors such as *P. concolor* may find it challenging to reach *B. oleae* larvae inside enlarged domesticated olives (Wang *et al.*, 2009c; Wang *et al.*, 2009d). Furthermore, it has be suggested that alternative frugivorous tephritid hosts may be better suited in natural environments where wild olives occur in various maturation stages simultaneously, thus offering host fruit for the host fly (Copeland *et al.*, 2004). As such, future studies should investigate what factors resulted in the olive fruit fly adapting to prefer cultivated olives over wild olives as hosts *i.e.* are there specific properties of cultivated olives that make them a more appealing and suitable host for the species? If so, what implications does this have for the current candidates of potential biocontrol agents such as the *Psyttalia* species, specifically *P. humilis* and *P. lounsburyi*?

Although this study has set the baseline to the knowledge of insects associated with wild and cultivated olives in the Western Cape, it is not without limitations. Firstly, in the survey of insects found associated with olive fruit, several specimens were only morphologically identified to the genus level *Ormyrus* sp., as this group is known to be challenging to identify using morphological characters. Moreover, the poor amplification performance of the standard DNA barcoding region for these specimens hampered the ability to at least verify these specimens as *Ormyrus* sp. at the sequence level.

Secondly, the clarification of the lifestyle of putative olive seed wasps should be viewed as preliminary, since the sample size (n = 50) and sampling area (three collection sites) did not allow for the survey of the full spectrum of diversity. This is particularly important for species *Eurytoma varicolor* and *Neochrysocharis formosus*, two species that were not detected using the multiplex panel. Moreover, the assumption of Neuenschwander (1982) and Silvestri (1913, 1914, 1915) with regards to *E. oleae* and *S. aethiopica* as phytophagous wasps are most probably true; however, since the status of *Ormyrus* sp. is unknown and was excluded from the species multiplex panel, the possibility of *Eurytoma oleae* and *Sycophila aethiopica* are parasitoids of *Ormyrus* sp. cannot totally be ruled out. However, *Ormyrus* sp. was found at very low frequency, not supporting the aforementioned possibility. As such, future studies should focus on extensive sampling by increasing the sample size, and following the distribution of these wasp species found in our previous survey.

Lastly, the sequencing, mapping and assembly of the mitochondrial genomes of the *Psyttalia* species was not straightforward, most probably due to the high AT content typical of insect mitogenomes. The A+T-rich regions of all *Psyttalia* species under investigation were not recovered, which may be due to the frequent A or T homopolymeric stretches. Moreover, the Ion Torrent platform does not perform well when sequencing homopolymeric stretches, with the error rate increasing with an increase in the size of homopolymeric stretches. Additionally, the user community for Ion Torrent is much smaller in comparison to other NGS platforms therefore assistance in problem-solving is limited, and other NGS platforms, such as Illumina sequencing should be explored.

This study comprises of the first molecular catalogue of insect species associated with wild and cultivated olive fruit in the Western Cape province of South Africa, including new DNA barcodes for several wasp species, as well as new publicly available high-resolution images that were previously non-existent. Moreover, the development of a DNA-based tool for the species identification of immature specimens in addition to the simultaneous elucidation of the lifestyles of OSW, provides the industry with a fast and easy method of detecting early infestations. This aspect is locally significant, as OSW are not known to occur in any other olive-producing world region. Besides generating the first mitochondrial genomes of three important parasitoid species of the olive fruit fly, this work contributes to the complex phylogeny of the family Braconidae and highlights the common occurrence of gene rearrangement within this group, further supporting the idea of using gene rearrangements as a phylogenetic tool in resolving complex phylogenetic relationships.

Supplementary data

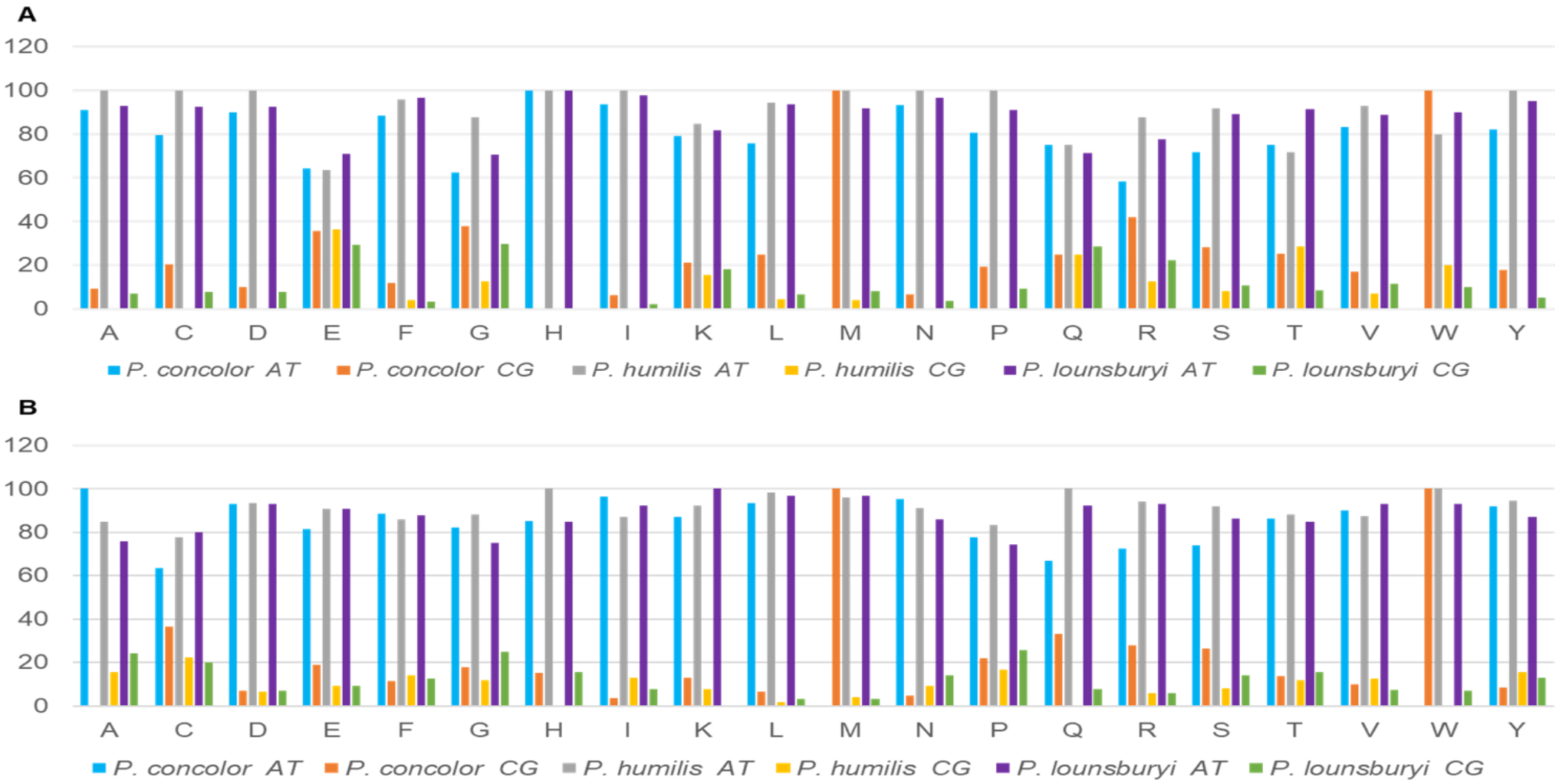

Figure S11. Relative usage of A, T, C, and G in the third codon position in protein-coding genes in the majority and minority strands of the partial mitochondrial genomes of *Psyttalia concolor*, *Psyttalia humilis* and *Psyttalia lounsburyi*. (A) A and T compared to G and C, for all amino acids in the majority strand; and (B) A and T compared to G and C, for all amino acids in the minority strand.

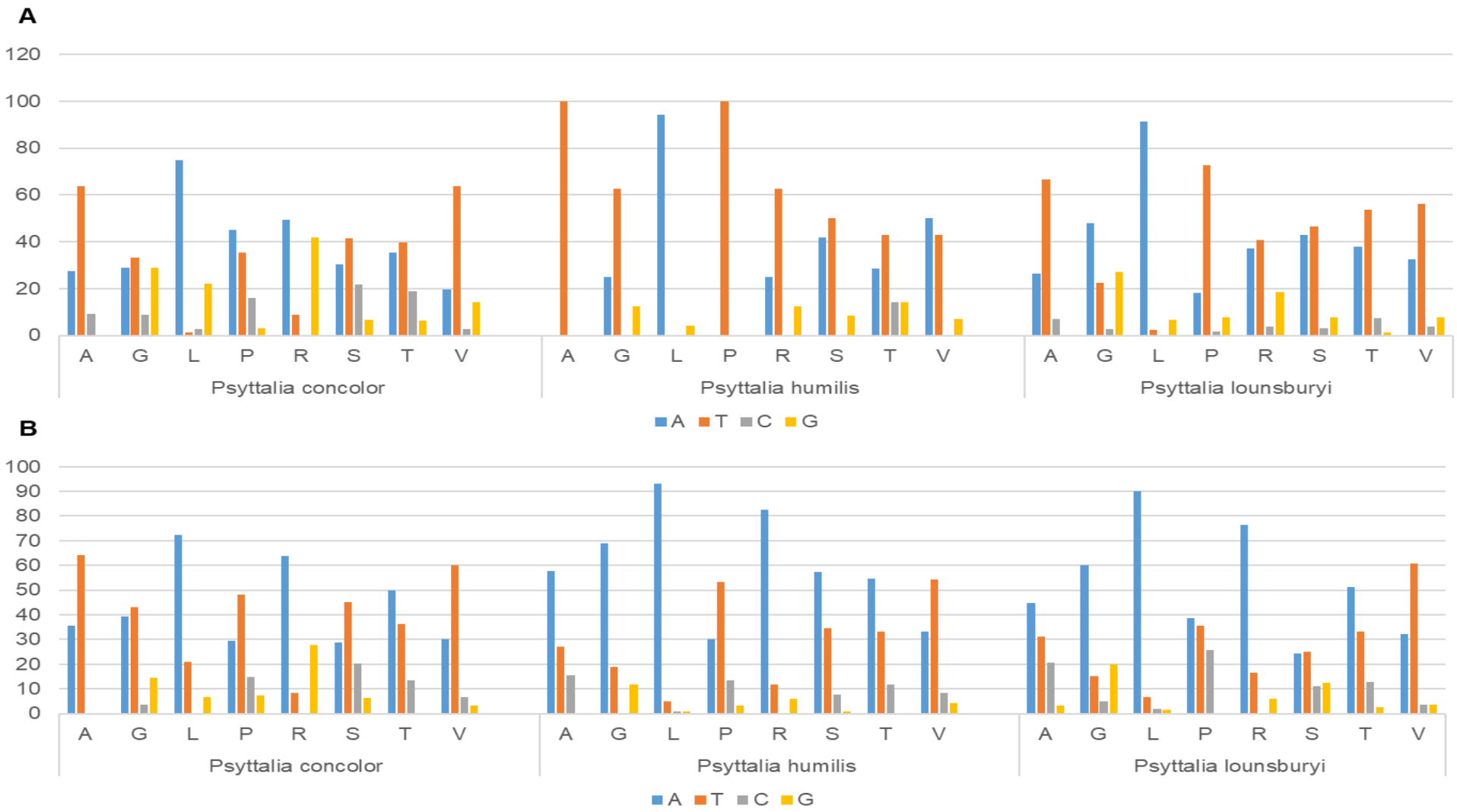

Figure S12. Relative usage of A, T, C, and G in the third codon position-coding genes in the majority and minority strands of the partial mitochondrial genomes of *Psyttalia concolor*, *Psyttalia humilis*, and *Psyttalia lounsburyi*. (A) A, T, C, and G or eight amino acids on the majority strand and (B) A, T, C, and G for eight amino acids on the minority strand. Amino acids are designated by the single-letter code.

Table S11. Specimen data of immature insect specimens found within the seed structure of wild and cultivated olives collected in three sites from two areas in the Western Cape province of South Africa, and tested for species identification using a species-specific multiplex PCR test.

Sample code	Species	Size (mm)	Collection date	Collection site	Area	Coordinates	Host
Lv01	*E. spermophilus*	3.23	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv03	*E. spermophilus*	2.45	01-Oct-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv04	*E. spermophilus*	3.17	14-Feb-18	Olyvenbosch Olive Farm	Paarl	33.6803 °S, 18.9075 °E	Cultivated
Lv10	*E. spermophilus*	3.52	18-Oct-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv11	*E. spermophilus*	3.53	18-Oct-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv16	*E. spermophilus*	2.74	14-Aug-18	Olyvenbosch Olive Farm	Paarl	33.6803 °S, 18.9075 °E	Wild
Lv17	*E. spermophilus*	2.80	20-Feb-18	Olyvenbosch Olive Farm	Paarl	33.6803 °S, 18.9075 °E	Cultivated
Lv18	*E. spermophilus*	2.80	09-Apr-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv19	*E. spermophilus*	2.80	20-Feb-18	Olyvenbosch Olive Farm	Paarl	33.6803 °S, 18.9075 °E	Cultivated
Lv20	*E. spermophilus*	2.80	20-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv21	*E. spermophilus*	2.80	09-Apr-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv22	*S. aethiopica*	2.80	20-Sep-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv23	*E. spermophilus*	2.80	20-Sep-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv24	*E. spermophilus*	2.80	20-Sep-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv25	*E. spermophilus*	2.80	24-Jan-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv26	*E. spermophilus*	2.80	27-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv27	*E. spermophilus*	2.80	24-Jan-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv28	*E. spermophilus*	2.80	30-Jan-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv29	*E. spermophilus*	2.80	30-Jan-18	Olyvenbosch Olive Farm	Paarl	33.6803 °S, 18.9075 °E	Cultivated
Lv30	*E. spermophilus*	2.80	14-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv31	*E. spermophilus*	2.80	06-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv32	*E. spermophilus*	2.80	06-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv33	*E. spermophilus*	2.80	09-Apr-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv34	*E. spermophilus*	2.80	14-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv35	*E. spermophilus*	2.80	30-Jan-18	Olyvenbosch Olive Farm	Paarl	33.6803 °S, 18.9075 °E	Cultivated
Lv36	*E. spermophilus*	2.80	06-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv37	*E. spermophilus*	2.80	27-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv38	*E. spermophilus*	2.80	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv39	*E. spermophilus*	2.80	18-Oct-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv40	*E. spermophilus*	2.80	18-Oct-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv43	*E. spermophilus*	2.80	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv46	*E. spermophilus*	2.80	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv47	*E. spermophilus*	2.80	18-Oct-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv48	*E. spermophilus*	2.80	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv52	*E. oleae*	1.86	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv56	*S. aethiopica*	1.51	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv57	*E. oleae*	1.79	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv59	*E. spermophilus*	2.17	14-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv60	*E. spermophilus*	2.05	24-Jan-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv61	*E. spermophilus*	1.88	18-Oct-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv63	*S. aethiopica*	1.23	14-Aug-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv68	*E. spermophilus*	2.03	20-Feb-18	Olyvenbosch Olive Farm	Paarl	33.6803 °S, 18.9075 °E	Cultivated
Lv69	*E. spermophilus*	2.45	20-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv70	*E. spermophilus*	2.05	30-Jan-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated
Lv72	*E. spermophilus*	2.35	20-Sep-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv73	*E. spermophilus*	1.87	20-Sep-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv75	*E. spermophilus*	1.83	20-Sep-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv76	*E. spermophilus*	1.74	20-Sep-18	Nietvoorbji	Stellenbosch	33. 9168 °S, 18.8598 °E	Wild
Lv84	*E. spermophilus*	2.89	14-Feb-18	Waterford Wine Estate	Stellenbosch	33.9953 °S, 18.8706 °E	Cultivated

Table S12. Polymerase Chain Reaction (PCR) cycling protocols and PCR, and sequencing (Seq) primers used for the Sanger sequencing of partial mitochondrial genomes of *Psyttalia concolor*. *Shorter versions of four of 18 segments sequenced.

Segment	Cycling protocol	Primer	Primer sequence (5´- 3´)	Use direction
S01	PAS2	TC16-023	TAG AAG TTT TAT TAG TGA TAA GTC	PCR-F, Seq-F
		TC14-172	TGT CTG ATT TAA GTA ATA AAC TG	PCR-R
S01*	PAS2	TC15-107	ATA AAA TTA GGA ATA TTT CCC TTT C	PCR-F, Seq-F
		TC14-172	TGT CTG ATT TAA GTA ATA AAC TG	PCR-R
S02	PAS1	TC14-160	ATA TCA TTA GGT GGT TTA CCT CC	PCR-F, Seq-F
		TC11-107	GGT ATC CCT AAC TCT AAC CG	PCR-R, Seq-R
S03	PAS1	11-102	GGT CAA CAA ATC ATA AAG ATA TTG G	PCR-F, Seq-F
		11-103	TAA ACT TCA GGG TGA CCA AAA AAT CA	PCR-R, Seq-R
S04	PAS1	TC11-104	GGG ACC CTG TTT TAT ATC AAC	PCR-F, Seq-F
		TC14-016	CGA TTA TCA ACA TCT AAT AAA CG	PCR-R, Seq-R
		TC14-171	CTG CTA TAA TAG CAA ATA CAG C	Seq-R
S05	PAS1	TC14-108	AGG GCA TCA ATG ATA CTG AAG	PCR-F, Seq-F
		TC14-107	AAT GTA ATT AAT AAA TGA CCA GC	PCR-R, Seq-R
S06	PAS2	TC15-039	TAT ATT AGC TCA TTT AGT TCC TC	PCR-F
		TC16-018	AAC AAA ATG TCA ATA TCA TGA GG	PCR-R, Seq-R
S06*	PAS1	TC15-039	TAT ATT AGC TCA TTT AGT TCC TC	PCR-F, Seq-F
		TC15-074	AAA AAA CAG ACC CAT AAA TTG AAT C	PCR-R, Seq-R
S07	PAS2	TC15-098	GAG TAT TCT GAA TCA TAT TTT TC	PCR-F, Seq-F
		TC15-082	GTT TAA TAA TGA AAA TAT TAA GAT G	PCR-R, Seq-R
S08	PAS2	TC15-110	AAT TCC AAT TAA AAT AAG TAT GAA C	PCR-F, Seq-F
		TC15-101	TTA CAA AAA GAG CCC AAA TTC C	PCR-R, Seq-R
S09	PAS1	TC15-043	AGT TAC TAA TGT TGA GGA ATG AAC	PCR-F, Seq-F
		TC15-044	TGA TTA CCT AAA GCC CAT GTA GAA G	PCR-R, Seq-R
S10	PAS3	TC16-004	GAT TAA ACT AGA AAT TAA GCT TCC	PCR-F, Seq-F
		TC15-083	AAA TTA TTA ACC CAC CAA TAA TTA C	PCR-R, Seq-R
S10*	PAS2	TC15-076	AAC GGT ATA ATC CAT AAG ACC C	PCR-F, Seq-F
		TC15-083	AAA TTA TTA ACC CAC CAA TAA TTA C	PCR-R, Seq-R
S11	PAS2	TC15-084	CTG GCT TGA ATT CGA TCA ATT TG	PCR-F
		TC15-077	TAT GAA TTA AAC GTA TTA ATC ACC C	PCR-R
		TC16-015	ATAGATACAGGAATTGTTTTTAAC	Seq-F
S12	PAS3	TC15-102	TTT AGT TAA TTT ACC TAC TCC TG	PCR-F, Seq-F
		TC15-103	CTG CTT GAG ACC CTA ATC AAG	PCR-R, Seq-R
S13	PAS1	TC15-045	CTC CTG TTC ATA TTC AAC CAG AAT G	PCR-F, Seq-F
		TC15-046	TGC TCA ATC TAT TTC TTA TGA AG	PCR-R
S14	PAS2	TC14-162	ATT CAG ATT CTC CTT CTG ATA AAT C	PCR-F, Seq-F
		TC14-164	ATA AAT TAC CTT AGG GAT AAC AGC	PCR-R, Seq-R
S15	PAS2	TC15-085	GGC TTA CGC CGA TCT TAA CTC	PCR-F, Seq-F
		TC15-086	TTA ATA TAG GTA CAT ATC GCC CGT C	PCR-R, Seq-R
S16	PAS2	TC15-104	TTA GAA TTA TTC TGA TAC AAA AGG	PCR-F, Seq-F
		TC15-105	ATG AAG TGC CTG AAT AAA AGG	PCR-R, Seq-R
S17	PAS2	TC15-088	AAT AAT AGG GTA TCT AAT CCT AGT	PCR-F
		TC16-022	ATT TTA TCC CTA ATT TAA CTG CC	PCR-R, Seq-R
S17*	PAS2	TC15-088	AAT AAT AGG GTA TCT AAT CCT AGT	PCR-F, Seq-F
		TC15-089	ATT TAA GCT TTT TGG TTC ATA CCC	PCR-R
S18	PAS2	TC15-106	AAT CCT TTT ATT CAG GCA CTT C	PCR-F, Seq-F
		TC15-109	CTC AAT TTA ACT CAA CTA ATA TAT C	PCR-R, Seq-R

Table S13. List of the partial and complete mitochondrial sequences used in the phylogenetic reconstruction of the family Braconidae including the new *Psyttalia concolor*, *Psyttalia humilis*, and *Psyttalia lounsburyi* and 28 other species publicly available. *Diadegma semiclausum* and *Enicosphilus* sp. (Ichneumonidae) were used as outgroups.

Species	Status	Lineage	GenBank	Reference	Size (bp)
Acanthormius sp.	Partial	Cyclostome	KF385867	Direct submission	13,051
Aphidius gifuensis	Partial	Cyclostome	GU097658	Wei et al. 2010	11,970
Afrocampsis griseosetosus	Partial	Non-cyclostome	KJ412474	Direct submission	10,104
Capitonius sp.	Partial	Non-cyclostome	KF385869	Direct submission	13,077
Cardiochiles fuscipennis	Partial	Non-cyclostome	KF385870	Direct submission	14, 390
Cotesia vestalis	Complete	Non-cyclostome	FJ154897	Wei et al. 2010	15, 543
Diachasmimorpha longicaudata	Partial	Cyclostome	GU097655	Wei et al. 2010	13, 850
Diadegma semiclausum	Complete	Non-cyclostome	EU871947	Wei et al. 2009	18, 728
Elasmosoma sp.	Partial	Non-cyclostome	KJ412470	Direct submission	12, 326
Enicospilus sp.	Partial	Non-cyclostome	FJ478177	Dowton et al. 2009	15, 300
Eumacrocentrus sp.	Partial	Non-cyclostome	KF385872	Direct submission	14, 080
Euurobracon breviterebrae	Partial	Cyclostome	KF385871	Direct submission	12, 957
Histeromerus sp.	Partial	Cyclostome	KF418765	Direct submission	13, 168
Homolobus sp.	Partial	Non-cyclostome	KF385873	Direct submission	13, 927
Ichneutes sp.	Partial	Non-cyclostome	KF385874	Direct submission	13, 092
Macrocentrus camphoraphilus	Partial	Non-cyclostome	GU097656	Wei et al. 2010	15, 801
Meteorus pulchricornis	Partial	Non-cyclostome	GU097657	Wei et al. 2010	10, 186
Mirax sp.	Partial	Non-cyclostome	KJ412471	Direct submission	13, 664
Pambolus sp.	Partial	Cyclostome	KF385875	Direct submission	13, 175
Paroligoneurus sp.	Partial	Non-cyclostome	KJ412472	Direct submission	13, 413
Phaenocarpa sp.	Partial	Cyclostome	KJ412475	Direct submission	9, 981
Phanerotoma flava	Partial	Non-cyclostome	GU097654	Wei et al. 2010	10, 171
Proterops sp.	Partial	Non-cyclostome	KJ412477	Direct submission	12, 883
Pselaphanus sp.	Partial	Non-cyclostome	KF385876	Direct submission	13, 204
Pseudognaptodon sp.	Partial	Cyclostome	KJ412473	Direct submission	13, 190
Psyttalia concolor	Partial	Cyclostome	Deposit upon acceptance	This study	15, 308
Psyttalia humilis	Partial	Cyclostome	Deposit upon acceptance	This study	15, 311
Psyttalia lounsburyi	Partial	Cyclostome	Deposit upon acceptance	This study	14, 982
Therophilus festivus	Partial	Non-cyclostome	KF385868	Direct submission	14, 216
Triraphius sp.	Partial	Cyclostome	KF385877	Direct submission	13, 162
Sigalphus bicolor	Partial	Non-cyclostome	KF385878	Direct submission	12, 744
Spathius agrili	Complete	Cyclostome	FJ387020	Wei et al. 2010	15, 425
Xiphozele sp.	Partial	Non-cyclostome	KJ412476	Direct submission	9, 160

Table S14. Nucleotide composition of the near-complete mitochondrial genomes of *Psyttalia concolor*, *Psyttalia humilis*, and *Psyttalia lounsburyi*. PCGs – protein-coding genes. AT-skew = (A-T)/(A+T); GC-skew = (G-C)/(G+C).

	Psyttalia concolor								*Psyttalia humilis*								*Psyttalia lounsburyi*							
Region	A%	C%	G%	T%	A+T%	G+C%	AT skew	GC skew	A%	C%	G%	T%	A+T%	G+C%	AT skew	GC skew	A%	C%	G%	T%	A+T%	G+C%	AT skew	GC skew
ND2	36.2	3.2	7.6	52.9	89.1	10.9	-0.19	0.41	37.0	3.4	7.5	52.0	89.0	11.0	-0.17	0.38	37.1	3.4	7.4	52.0	89.1	10.9	-0.17	0.37
COI	30.2	9.8	15.8	44.0	74.4	25.6	-0.19	0.23	30.6	9.4	15.9	44.1	74.7	25.3	-0.18	0.26	30.7	10.2	15.1	43.9	74.7	25.3	-0.18	0.19
COII	36.1	7.3	12.0	44.4	80.6	19.4	-0.10	0.24	35.3	7.0	12.6	45.1	80.4	19.6	-0.12	0.29	35.6	7.6	11.3	45.5	81.1	18.9	-0.12	0.20
ATP8	39.1	5.1	5.8	50.0	89.1	10.9	-0.12	0.06	37.8	4.5	5.1	52.6	90.4	9.6	-0.16	0.06	36.5	5.1	4.5	53.8	90.4	9.6	-0.19	-0.06
ATP6	33.5	7.8	8.3	50.4	83.9	16.1	-0.20	0.03	34.1	8.0	7.8	50.1	84.2	15.8	-0.19	-0.01	32.2	8.6	8.7	50.6	82.8	17.2	-0.22	0.01
COIII	31.3	8.6	14.7	45.4	76.7	23.3	-0.18	0.26	30.7	8.7	15.6	45.0	75.7	24.3	-0.19	0.28	30.4	8.6	15.7	45.2	75.7	24.3	-0.20	0.29
ND3	32.7	3.9	10.1	53.2	86.0	14.0	-0.24	0.44	33.1	3.9	10.1	53.0	86.0	14.0	-0.23	0.44	33.1	4.8	10.6	51.6	84.7	15.3	-0.22	0.38
ND5	45.4	6.2	9.5	38.9	84.3	15.7	0.08	0.21	44.8	6.5	9.7	39.1	83.9	16.1	0.07	0.20	44.7	6.3	10.0	39.0	83.7	16.3	0.07	0.23
ND4	44.8	7	10.1	38.1	82.9	17.1	0.08	0.18	44.9	6.9	9.8	38.4	83.3	16.7	0.08	0.17	44.4	7.3	9.9	38.4	82.8	17.2	0.07	0.15
ND4L	49.5	6.4	6.1	38	87.5	12.5	0.13	-0.02	50.2	6.4	5.7	37.7	87.9	12.1	0.14	-0.06	49.2	6.7	6.4	37.7	86.9	13.1	0.13	-0.02
ND6	38.7	3.7	6.4	51.2	89.9	10.1	-0.14	0.27	38.3	4.2	6.9	50.6	88.9	11.1	-0.14	0.24	39.0	4.2	6.9	49.9	88.9	11.1	-0.12	0.24
CytB	33.2	8.7	11.6	46.5	79.7	20.3	-0.17	0.14	33.2	8.8	12.2	45.8	79.0	21.0	-0.16	0.16	33.5	9.4	11.9	45.2	78.7	21.3	-0.15	0.12
ND1	45.0	9.1	9.9	36	81.0	19.0	0.11	0.04	44.3	9.1	10.4	36.3	80.5	19.5	0.10	0.07	44.3	9.3	10.2	36.3	80.5	19.5	0.10	0.05
srRNA	39.9	4.3	5.2	50.6	90.5	9.5	-0.12	0.09	39.5	4.3	5.6	50.3	90.1	9.9	-0.12	0.13	39.5	4.6	4.9	50.8	90.4	9.6	-0.13	0.03
lrRNA	41.4	5.4	6.2	47.0	88.4	11.6	-0.06	0.07	41.2	5.4	6.1	47.2	88.4	11.6	-0.07	0.06	42.3	5.2	6.9	43.1	87.6	12.4	-0.01	0.14
PCGs	38.1	7.1	10.7	44.1	82.2	17.8	-0.07	0.20	38.0	7.1	10.8	44.0	82.0	18.0	-0.07	0.21	37.9	7.5	10.8	43.9	81.8	18.2	-0.07	0.18
tRNAs	42.9	5.9	7.2	44	87.0	13.0	-0.01	0.10	42.6	5.8	7.1	43.6	87.2	12.8	-0.01	0.10	42.8	5.4	7.9	43.8	86.7	13.3	-0.01	0.19
rRNAs	40.8	5.0	5.8	48.3	89.1	10.9	-0.08	0.07	40.6	5.0	5.9	48.4	89.0	11.0	-0.09	0.08	41.3	5.0	6.2	46.0	88.7	11.3	-0.05	0.11
Complete mtDNA	39.4	6.5	9.5	44.6	84.0	16.0	-0.06	0.19	39.3	6.5	9.6	44.5	83.9	16.1	-0.06	0.19	39.0	6.8	9.8	44.4	83.4	16.6	-0.06	0.18

Table S15. AT- and GC-skews in the protein-coding genes, tRNAs, rRNAs and AT-rich region of partial and complete mitochondrial genomes of 31 Braconidae species. AT-skew = (A-T)/(A+T); GC-skew = (G-C)/(G+C).

	Whole sequence		PCGs		tRNAs		rRNAs	
Species	AT-skew	GC-skew	AT-skew	GC-skew	AT-skew	GC-skew	AT-skew	GC-skew
Acanthormius sp.	-0.11	0.19	-0.11	0.20	-0.02	0.13	-0.14	0.04
Afrocampsis griseosetosus	0.44	0.31	-0.11	0.34	-0.04	0.35	0.05	0.05
Aphidius gifuensis	-0.06	0.05	-0.06	0.07	-0.02	0.00	-0.07	-0.10
Capitonius sp.	-0.07	0.19	-0.07	0.22	-0.01	0.14	-0.09	-0.09
Cardiochiles fuscipennis	-0.07	0.18	-0.08	0.22	-0.05	0.10	-0.06	0.01
Cotesia vestalis	-0.09	0.10	-0.11	0.12	-0.03	0.11	-0.03	-0.11
Diachasmimorpha longicaudata	0.09	0.19	-0.10	0.21	-0.03	0.17	-0.02	0.05
Diadegma semiclausum	0.01	-0.20	0.01	-0.18	0.00	-0.11	-0.02	-0.31
Elasmosoma sp.	-0.12	0.38	-0.14	0.40	0.05	0.24	-0.07	0.16
Enicospilus sp.	-0.02	-0.18	-0.02	-0.16	0.00	-0.10	-0.04	-0.29
Eumacrocentrus sp.	-0.01	0.05	-0.02	0.07	0.01	0.07	0.03	-0.17
Euurobracon breviterebrae	-0.11	0.37	-0.13	0.39	-0.03	0.32	-0.09	0.17
Histeromerus sp.	-0.06	0.16	-0.06	0.19	-0.01	0.04	-0.09	-0.02
Homolobus sp.	-0.06	0.10	-0.06	0.10	0.00	0.09	-0.06	-0.06
Ichneutes sp.	-0.06	0.21	-0.06	0.24	-0.03	0.10	-0.09	-0.01
Macrocentrus camphoraphilus	-0.05	0.10	-0.06	0.13	-0.01	0.02	-0.04	-0.11
Meteorus pulchricornis	-0.06	0.14	-0.06	0.16	-0.02	0.15	-	-
Mirax sp.	-0.07	0.19	-0.07	0.02	-0.04	0.22	-0.09	-0.06
Pambolus sp.	-0.09	0.16	-0.10	0.17	-0.05	0.09	-0.08	0.00
Paroligoneurus sp.	-0.12	0.22	-0.12	0.25	-0.04	0.08	-0.15	0.00
Phaenocarpa sp.	-0.09	0.11	-0.10	0.14	0.00	0.17	-0.08	-0.08
Phanerotoma flava	-0.07	0.28	-0.07	0.29	-0.01	0.15	-	-
Proterops sp.	0.06	-0.15	0.07	-0.14	0.03	0.00	0.06	-0.15
Pselaphanus sp.	-0.03	0.04	-0.03	0.08	0.02	0.01	-0.05	-0.17
Pseudognaptodon sp.	-0.02	0.03	-0.02	0.04	-0.03	0.11	-0.03	-0.18
Psyttalia concolor	-0.06	0.19	-0.07	0.20	-0.01	0.10	-0.08	0.07
Psyttalia humilis	-0.06	0.19	-0.07	0.21	-0.01	0.10	-0.09	0.08
Psyttalia lounsburyi	-0.06	0.18	-0.07	0.18	-0.01	0.19	-0.05	0.11
Therophilus festivus	-0.03	0.02	-0.02	0.05	0.01	0.01	-0.06	-0.13
Triraphius sp.	-0.12	0.19	-0.12	0.21	-0.06	0.18	-0.17	-0.11
Sigalphus bicolor	-0.03	0.00	-0.02	0.02	-0.02	0.07	-0.04	-0.18
Spathius agrili	-0.07	0.19	-0.07	0.20	-0.04	0.13	-0.12	0.01
Xiphozele sp.	-0.01	-0.05	-0.02	-0.02	0.02	-0.02	0.03	-0.27
Average	-0.04	0.13	-0.07	0.14	-0.01	0.10	-0.06	-0.06

Table S16. Usage of start and stop codons in 13 mitochondrial protein-coding genes of 31 partial and complete mitochondrial sequences of the family Braconidae. Undetermined start and stop codons are represented by (-).

	ATP6		*ATP8*		*COX1*		*COX2*		*COX3*		*CytB*		*ND1*	
Species	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**
Acanthormius sp.	ATG	TAA	ATT	TAA	-	TAA	ATT	TAA	ATG	TAA	ATG	TAA	ATC	TAA
Aphidius gifuensis	ATG	TA	ATT	TAA	-	TAA	ATT	TAA	ATT	T	ATG	TAG	ATT	TAA
Afrocampsis griseosetosus	ATT	TAA	ATA	TAA	-	TAG	ATG	TAA	ATA	TAA	ATT	TAA	ATT	TAA
Capitonius sp.	ATA	TAA	ATA	TAA	-	T	ATT	TAA	ATG	TAA	ATG	TAG	ATT	TAA
Cardiochiles fuscipennis	ATA	TAA	ATA	TAA	ATG	TAA	ATT	TAG	ATG	TAA	ATG	TAG	ATT	TAA
Cotesia vestalis	ATG	TAA	ATT	TAA	ATG	TAA	ATT	TAA	ATG	TAA	ATG	TAA	ATT	TAA
Diachasmimorpha longicaudata	ATG	TAA	ATA	TAA	-	TAA	ATT	TAA	ATG	TAA	ATG	TAG	ATT	TAA
Diadegma semiclausum	ATG	TA	ATC	TAA	ATT	TAA	ATA	T	ATA	TAA	ATG	TAG	ATT	TAA
Elasmosoma sp.	ATA	TAA	ATT	TAA	-	TAG	ATG	TAG	ATG	TAA	ATT	TAG	ATT	TAA
Enicospilus sp.	ATG	TAA	ATT	TAA	ATT	TAA	ATA	T	ATG	T	ATG	T	ATA	T
Eumacrocentrus sp.	ATA	TAA	ATT	TAA	-	TAA	ATT	TAA	ATT	TAG	ATT	TAG	ATT	TAA
Euurobracon breviterebrae	ATA	TAA	ATT	TAA	-	TA	ATT	TAA	ATT	TAG	ATT	TAG	ATT	TAA
Histeromerus sp.	ATA	TAA	ATA	TAA	-	TAA	ATT	TAA	ATA	TAA	ATG	TAA	ATT	TAG
Homolobus sp.	ATA	TAA	ATA	TAA	-	TAA	ATT	TAA	ATG	TAA	ATG	TAA	ATA	TAA
Ichneutes sp.	ATA	TAA	ATA	TAA	-	TAA	ATT	TAA	ATA	TAA	ATG	TAA	ATT	TA
Macrocentrus camphoraphilus	ATG	TAA	ATT	TAA	ATG	TAA	ATT	TAA	ATT	TAA	ATG	TAA	ATA	TAA
Meteorus pulchricornis	ATA	TAA	ATA	TAA	-	TAA	ATA	TAA	ATG	TAA	-	TAA	ATA	TAA
Mirax sp.	ATA	TAA	ATA	TAA	-	TAA	ATT	TAA	ATG	TAA	ATG	TAA	ATA	TAA
Pambolus sp.	ATG	TAA	ATA	TAA	-	TAA	ATT	TAA	ATA	TAA	ATG	TAG	ATT	TAA
Paroligoneurus sp.	ATA	TAA	ATA	TAA	-	TAA	ATT	TAA	ATG	TAA	ATG	TAG	ATT	TAA
Phaenocarpa sp.	ATA	TAA	ATT	TAA	-	TAA	ATT	TAA	ATT	TAA	ATT	TAA	ATT	TAA
Phanerotoma flava	ATA	TAA	ATA	TAA	ATG	TAA	ATT	TAA	ATT	TAA	ATT	-	-	-
Proterops sp.	ATA	TAA	ATA	TAA	-	TAA	ATT	TAA	ATG	T	ATG	TAA	ATA	TAA
Pselaphanus sp.	ATG	TAA	ATT	TAA	ATG	TAA	ATT	TAA	ATG	TAA	ATT	TAA	ATT	TAA
Pseudognaptodon sp.	ATT	TAA	ATT	TAA	-	TAA	ATT	TAA	ATG	TAA	ATG	TAA	ATT	TAA
Psyttalia concolor	ATT	TAA	ATA	TAA	ATG	T	ATA	TAA	ATG	TAA	ATG	T	ATT	T
Psyttalia humilis	ATT	TAA	ATA	TAA	ATG	TAA	ATA	TAA	ATG	TAA	ATG	TAA	ATT	TAA
Psyttalia lounsburyi	ATT	TAA	ATA	TAA	ATG	TAA	ATA	TAA	ATG	TAA	ATG	TAA	ATT	TAA
Therophilus festivus	ATG	TAA	ATA	TAA	ATG	TA	ATT	TAA	ATA	TAA	ATG	TAG	ATT	TAA
Triraphius sp.	ATT	TAA	ATT	TAG	-	TAA	ATT	TAG	ATG	TAA	ATG	TAA	ATA	TAA
Sigalphus bicolor	ATA	TAA	ATC	TAA	-	TA	ATT	TAA	ATG	T	ATG	TAG	ATT	TAA
Spathius agrili	ATG	TAA	ATT	TAA	ATG	TAA	ATA	TAA	ATG	TAA	ATG	TAA	ATT	TAA
Xiphozele sp.	ATA	TAA	ATA	TAA	-	T	ATT	TAA	ATT	-	ATG	TAG	ATT	TAA

Table S17. Cont.

	ND2		*ND3*		*ND4*		*ND4L*		*ND5*		*ND6*	
Species	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**	**Start**	**Stop**
Acanthormius sp.	-	-	ATT	TAA	ATG	TAA	ATT	TAA	ATT	TAA	ATG	TAA
Aphidius gifuensis	-	-	ATA	TAG	ATG	-	ATT	TAA	ATT	TAA	ATT	T
Afrocampsis griseosetosus	-	-	-	-	ATG	-	ATT	TAA	-	-	ATG	TAA
Capitonius sp.	-	-	ATT	TAG	ATG	TAA	ATT	TAA	ATT	TAA	ATA	TAA
Cardiochiles fuscipennis	-	TAA	ATT	TAG	ATG	T	ATA	TAA	ATT	TA	ATG	TAG
Cotesia vestalis	ATT	TAA	ATA	TAA	ATG	TAA	ATT	TAA	ATT	TAA	ATG	TAA
Diachasmimorpha longicaudata	-	-	ATA	TAA	ATG	TAA	ATT	TAA	ATT	T	ATG	TAA
Diadegma semiclausum	ATA	TAA	ATT	TAG	ATG	TAA	ATT	TAA	ATG	TAA	ATG	T
Elasmosoma sp.	-	-	ATA	TAG	ATG	T	ATT	TAA	ATC	TAA	ATG	TAA
Enicospilus sp.	ATA	T	ATT	TAA	ATA	TAA	ATT	TAA	ATT	TAA	ATT	T
Eumacrocentrus sp.	-	-	ATT	TAA	ATT	TAA	ATA	TAA	ATT	TAA	ATG	TAA
Euurobracon breviterebrae	-	-	ATT	TAA	ATT	TAA	ATA	TAA	ATT	TAA	ATG	TAA
Histeromerus sp.	-	-	ATT	TAA	ATG	TAA	ATT	TAA	ATA	TAA	ATG	TAA
Homolobus sp.	-	-	ATT	TAA	ATG	TA	-	-	ATA	TA	ATT	TAA
Ichneutes sp.	-	-	ATA	TAA	ATT	TA	ATT	TAG	ATT	TAA	ATG	TAA
Macrocentrus camphoraphilus	-	-	ATA	TAA	ATC	TAA	ATT	TAA	ATA	TAA	ATT	TAA
Meteorus pulchricornis	-	-	ATA	TAA	ATG	T	-	TAA	ATT	TAA	-	-
Mirax sp.	-	-	ATA	TAG	ATA	TAA	ATA	TAA	ATT	TAA	ATT	TAA
Pambolus sp.	-	-	ATA	TAG	ATG	TAA	ATT	TAA	ATA	TA	ATG	TAA
Paroligoneurus sp.	-	-	ATT	TAA	ATG	T	ATT	TAA	ATT	T	ATG	TAA
Phaenocarpa sp.	-	-	ATT	TAA	-	-	-	-	-	T	-	-
Phanerotoma flava	ATT	TAA	ATA	TAA	ATG	TAA	ATT	TAA	ATA	TAA	ATA	TAA
Proterops sp.	-	-	ATT	TAA	ATG	TAA	ATT	TAA	ATT	T	ATG	TAA
Pselaphanus sp.	-	-	ATT	TAA	ATG	TAA	ATT	TAA	ATT	TAA	ATT	TAA
Pseudognaptodon sp.	-	-	ATT	TAG	ATG	TAA	ATT	TAA	ATT	T	ATG	TAA
Psyttalia concolor	ATA	T	ATT	T	ATG	TAA	ATT	TAA	ATA	T	ATG	TA
Psyttalia humilis	ATA	TAA	ATT	TAG	ATG	TAA	ATT	TAA	ATA	TAA	ATG	TAA
Psyttalia lounsburyi	ATA	TAA	ATT	TAG	ATG	TAA	ATT	TAA	ATA	TAA	ATG	TAA
Therophilus festivus	-	-	ATA	TAA	ATG	T	ATT	TAA	ATT	T	ATA	TAA
Triraphius sp.	-	-	ATT	TAA	ATT	TAA	ATT	T	ATT	T	ATG	TAA
Sigalphus bicolor	-	-	ATT	TAA	ATG	TAA	ATT	TAA	ATC	TAA	ATT	TAA
Spathius agrili	ATT	TAA	ATA	TAA	ATG	TAA	ATT	TAA	ATT	T	ATG	TAA
Xiphozele sp.	-	-	-	-	ATG	T	ATT	TAA	-	-	ATA	TAA

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