NRC Research Press

ARTICLE

Barcoding of parasitoid wasps (Braconidae and Chalcidoidea) associated with wild and cultivated olives in the Western Cape of South Africa[1]

Chante Powell, Virgilio Caleca, Martina Sinno, Michaela van Staden, Simon van Noort, Clint Rhode, Elleunorah Allsopp, and Barbara van Asch

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

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 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 to further investigate the biology and ecology of these species.

Key words: Braconidae, Chalcidoidea, DNA barcoding, olives, species identification.

Résumé : Les olives sauvages et cultivées abritent et partagent une grande diversité d'insectes, dont certains sont considérés comme des ravageurs, comme la mouche de l'olive. Il existe une riche diversité de parasitoïdes et de guêpes séminivores spécialisés associés aux oliviers en Afrique subsaharienne, dont plusieurs espèces indigènes qui auraient co-évolué avec leurs hôtes. Bien que des enquêtes entomologiques historiques aient rapporté la diversité des guêpes de l'olive dans la province du Cap-Occidental en Afrique du Sud, aucune étude approfondie n'a été réalisée dans cette région depuis l'avènement de méthodes moléculaires. Dans ce travail, une approche double combinant des méthodes morphologiques et basées sur l'ADN ont été employées pour identifier des spécimens adultes élevés sur des olives. Quatre espèces de Braconidae et six espèces de Chalcidoidea ont été identifiées sur la base de la morphologie et des méthodes de codage à barres de l'ADN ont été employées pour étudier la conspécificité chez des individus choisis au hasard parmi des spécimens représentatifs. Les identifications morphologiques étaient en accord avec les données moléculaires car les arbres NJ et ML ont correctement placé les séquences de chacune des espèces, soit en fonction du genre ou de l'espèce selon la couverture des taxons, et les distances génétiques ont fortement supporté la conspécificité. Aucune évidence claire de diversité cryptique n'a été trouvée. Globalement, les niveaux d'infestation des graines et les taux de parasitisme étaient plus élevés chez les olives sauvages que chez les olives cultivées, atteignant un sommet chez l'*Eupelmus spermophilus* et l'*Utetes africanus*. Ces résultats pourront servir à la détection moléculaire précoce des larves de guêpes dans les olives et pour étudier la biologie et l'écologie de ces espèces. [Traduit par la Rédaction]

Mots-clés : Braconidae, Chalcidoidea, codage à barres de l'ADN, olives, identification des espèces.

Received 31 March 2018. Accepted 8 October 2018.

Corresponding Editor: Monica Mwale.

C. Powell, M. van Staden, C. Rhode, and B. van Asch. Department of Genetics, University of Stellenbosch, Stellenbosch, South Africa.
V. Caleca. Department of Agricultural and Forestry Sciences, University of Palermo, Italy.
M. Sinno. Department of Agricultural Sciences, University of Naples "Federico II", Naples, Italy.
S. van Noort. Division of Entomology, Department of Natural History, Iziko South African Museum, Cape Town, South Africa.
E. Allsopp. Agricultural Research Council, Infruitec-Nietvoorbij, Stellenbosch, South Africa.
Corresponding author: Barbara van Asch (email: bva@sun.ac.za).

[1]This paper is part of a special issue entitled "Trends in DNA Barcoding and Metabarcoding".

Copyright remains with the author(s) or their institution(s). Permission for reuse (free in most cases) can be obtained from RightsLink.

 Published at www.nrcresearchpress.com/gen on 26 October 2018.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

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 (Silvestri 1915; Copeland et al. 2004; Mkize et al. 2008). 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 producer of olives, 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 (Silvestri 1913, 1915; Neuenschwander 1982).

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 (Zohary 1994; Nardi et al. 2005, 2010; Daane and Johnson 2010). *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 (Munro 1926, 1984; Mkize et al. 2008). 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 (Giacalone 2011; Caleca et al. 2015, 2017).

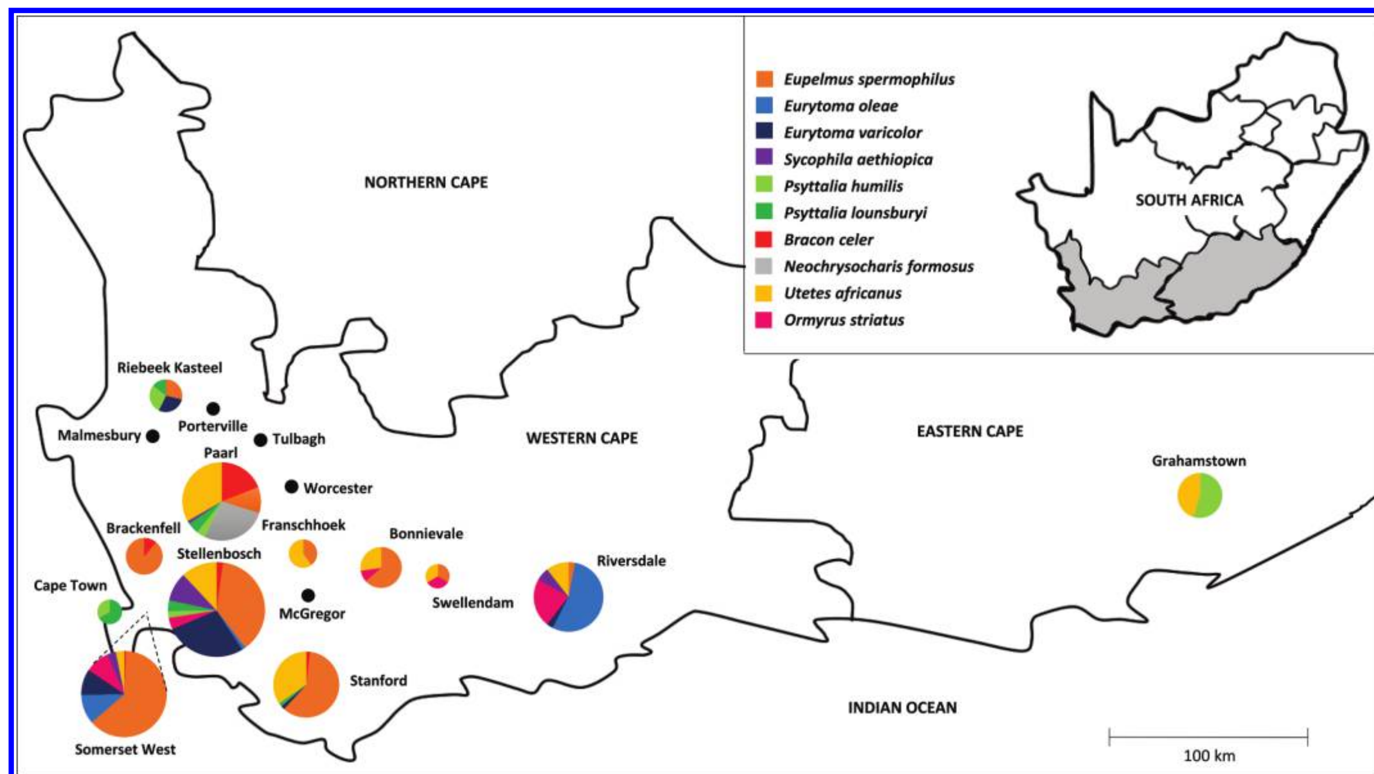
The potential utility of parasitoid wasps for the biological control of *B. oleae* in the Mediterranean basin, where it causes significant damage (Daane and Johnson 2010), and regions with similar climates such as the Middle East and California, where invasion has occurred more recently (Rice et al. 2003; Ramezani et al. 2015), 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) (Silvestri 1939; Caleca et al. 2017) and was purposefully imported into most of southern Europe for the control of *B. oleae* (Hoelmer et al. 2011; Borowiec et al. 2012).

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 (Silvestri 1913, 1915; Neuenschwander 1982). 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 hymenopterans 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 (Rowley et al. 2007; Al Khatib et al. 2014). 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 and Hebert 2007) that will increasingly allow for the assignment of unknown specimens to morphologically determined taxa, the dis-

Fig. 1. Areas of collection of wild and cultivated olives in the Western (16) and the 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.



Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

crimination of cryptic species, and the elucidation of synonymies (Hebert and 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 study 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.

Material and methods

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, between March and October 2016 (Fig. 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 unsprayed 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.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

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 et al. (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 Bouček et al. (1981) and Nieves-Aldrey et al. (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 Microsystems, 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[2]). DNA sequences were not generated from the deposited specimens but from other specimens equally representative of each species, according to morphological identifications.

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 et al. 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[2]).

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.

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[2]). 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)

[2]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2018-0068.

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.

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.

Results

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[2]). 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%). 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 Figs. 2–5. 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 2583 cultivated olives in three areas (Franschhoek, Paarl, and Stellenbosch): *E. spermophilus* (*n* = 4), *B. celer* (*n* = 2), and *P. lounsburyi* (*n* = 1) (Fig. 6).

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[2]). 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[2]). 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[2]). 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[2]).

For an overview of the current taxonomic coverage of Braconidae and Chalcidoidea, two separate family trees were constructed, with posterior condensation of species clusters into single branches (Figs. 7 and 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[2]–S7[2]). 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

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

Fig. 2. (A) *Bracon celer* female; (B) *Bracon celer* male; (C) *Psyttalia humilis* female; (D) *Psyttalia humilis* male.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

reference to the relevant topological differences in the text.

Braconidae

Bracon celer Szépligeti (Figs. 2A–2B) 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* (Fig. S1[2]).

Psyttalia humilis (Silvestri) (Figs. 2C–2D) was reared exclusively from wild olives in five areas, and represented 13.5% of all braconids, whereas *P. lounsburyi* (Figs. 3A–3B) 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[2]). 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[2]). 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[2]). 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.,

Fig. 3. (A) *Psyttalia lounsburyi* female; (B) *Psyttalia lounsburyi* male; (C) *Utetes africanus*, female; (D) *Utetes africanus* male.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

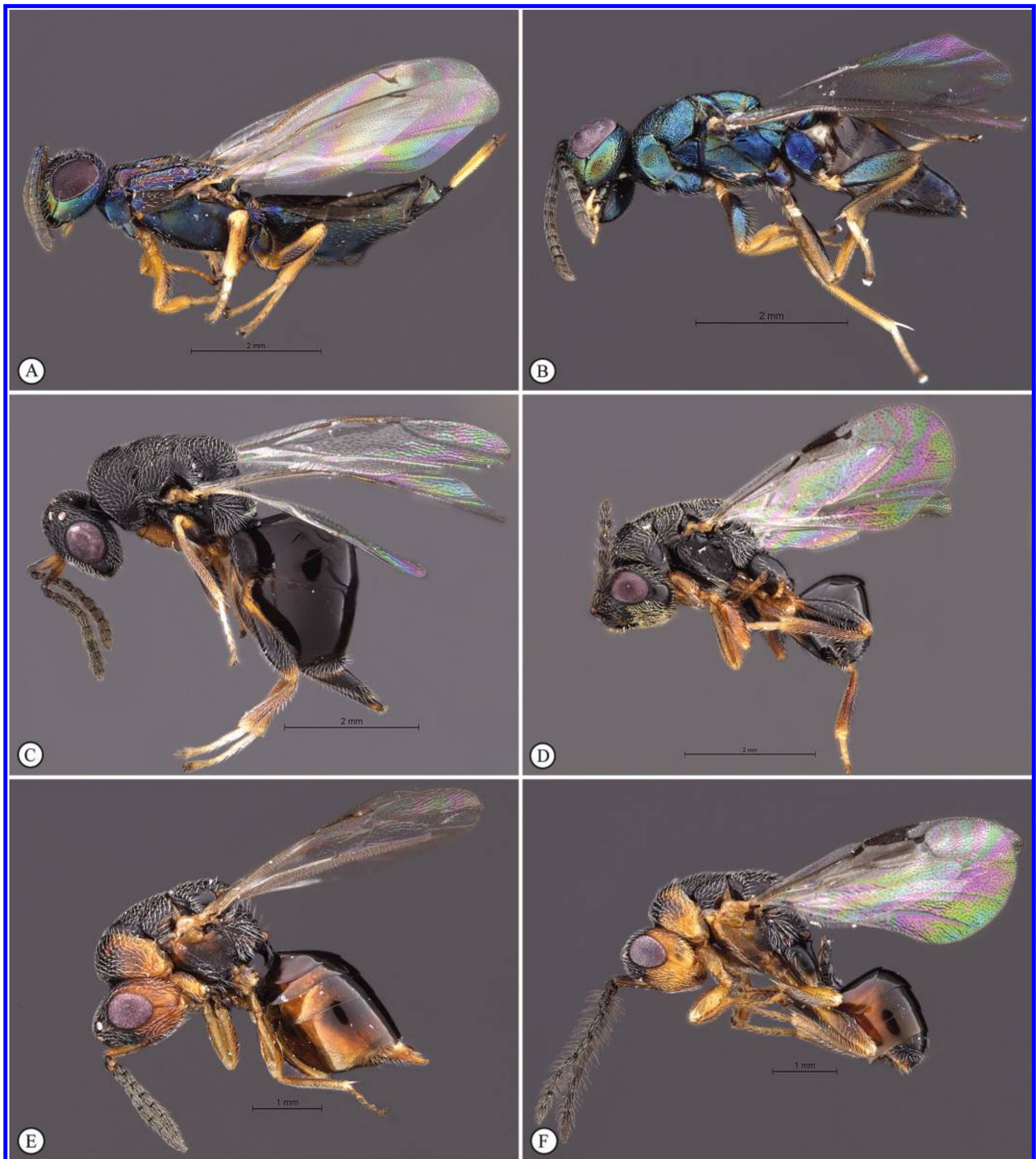
high genetic distances) were found; therefore, the public dataset did not seem to include sequences incorrectly assigned to species.

Utetes africanus (Szépligeti) (Figs. 3C–3D) was the most abundantly reared braconid (62.5%), and was exclusively found in wild olives in nine areas (Table S4[2]). 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 (Fig. S3[2]). 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 (Fig. S3[2]). 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[2]), 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%).

Chalcidoidea

Eupelmus spermophilus Silvestri (Figs. 4A–4B) 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[2]). The NJ and ML trees of the genus *Eupelmus* included 99 sequences distributed among 37 species, with 18 species represented by a single sequence (Fig. S4[2]). 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[2]). 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

Fig. 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.

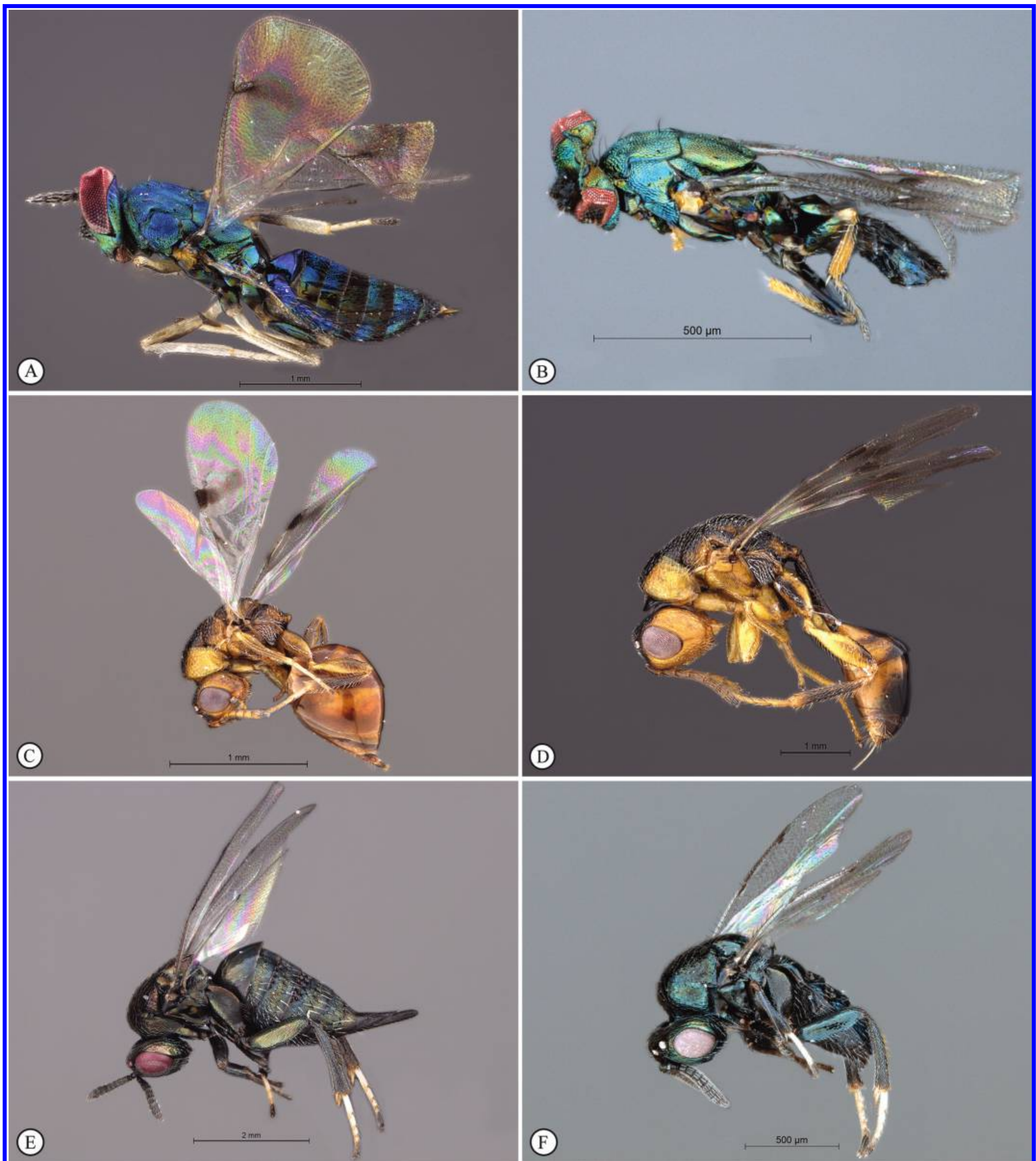


Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

E. spermophilus/*E. azureus* and 7.8% for the lowest pair *E. minozonus*/*E. urozonus*.

Eurytoma oleae Silvestri (Figs. 4C–4D) represented 9.1% of the total chalcids, and was reared exclusively from wild olives in three areas (Table S4[2]). The highest IF was found in Riversdale (1.89%), whereas the average was 0.10% in the other areas. *Eurytoma varicolor* Silvestri (Figs. 4E–4F) was reared exclusively from wild olives in five areas, and represented 21.4% of the total chalcids (Table S4[2]). The NJ and ML trees for the genus *Eurytoma*

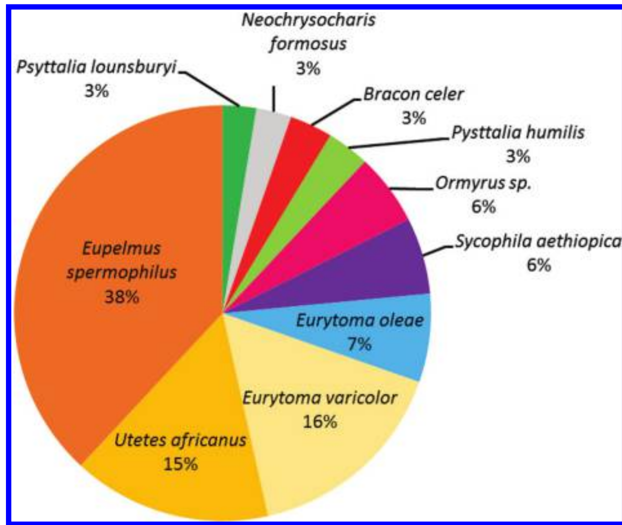
Fig. 5. (A) *Neochrysocharis formosus* female; (B) *Neochrysocharis formosus* male; (C) *Sycophila aethiopica* female; (D) *Sycophila aethiopica* male; (E) *Ormyrus* sp. female; (F) *Ormyrus* sp. male.



Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

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) (Fig. S5[2]). 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[2]).

Fig. 6. Relative proportions of adult braconid and chalcid wasps reared from wild and cultivated olives collected in 16 areas in the Western Cape Province and one area in the Eastern Cape Province of South Africa.



Sycophila aethiopica (Silvestri) (Figs. 5C–5D) was reared exclusively from wild olives in four areas, and represented 7.9% of the total chalcids (Table S4[2]). 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 (Fig. S6[2]).

Neochrysocharis formosus (Westwood) (Figs. 5A–5B) was reared exclusively from wild olives in Paarl (n = 23), and represented the lowest proportion (3.6%) of the total chalcids (Table S4[2]). 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 (Fig. S7[2]). Genetic distance estimates showed that the maximum divergence between the three *N. formosus* sequences was 12.9% (Table S10[2]). 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. (Figs. 5E–5F) was reared exclusively from wild olives in five areas, and represented 7.4% of the total chalcids (Table S4[2]). 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%) (Fig. S8[2]). *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) (Figs. S9[2]–S10[2]).

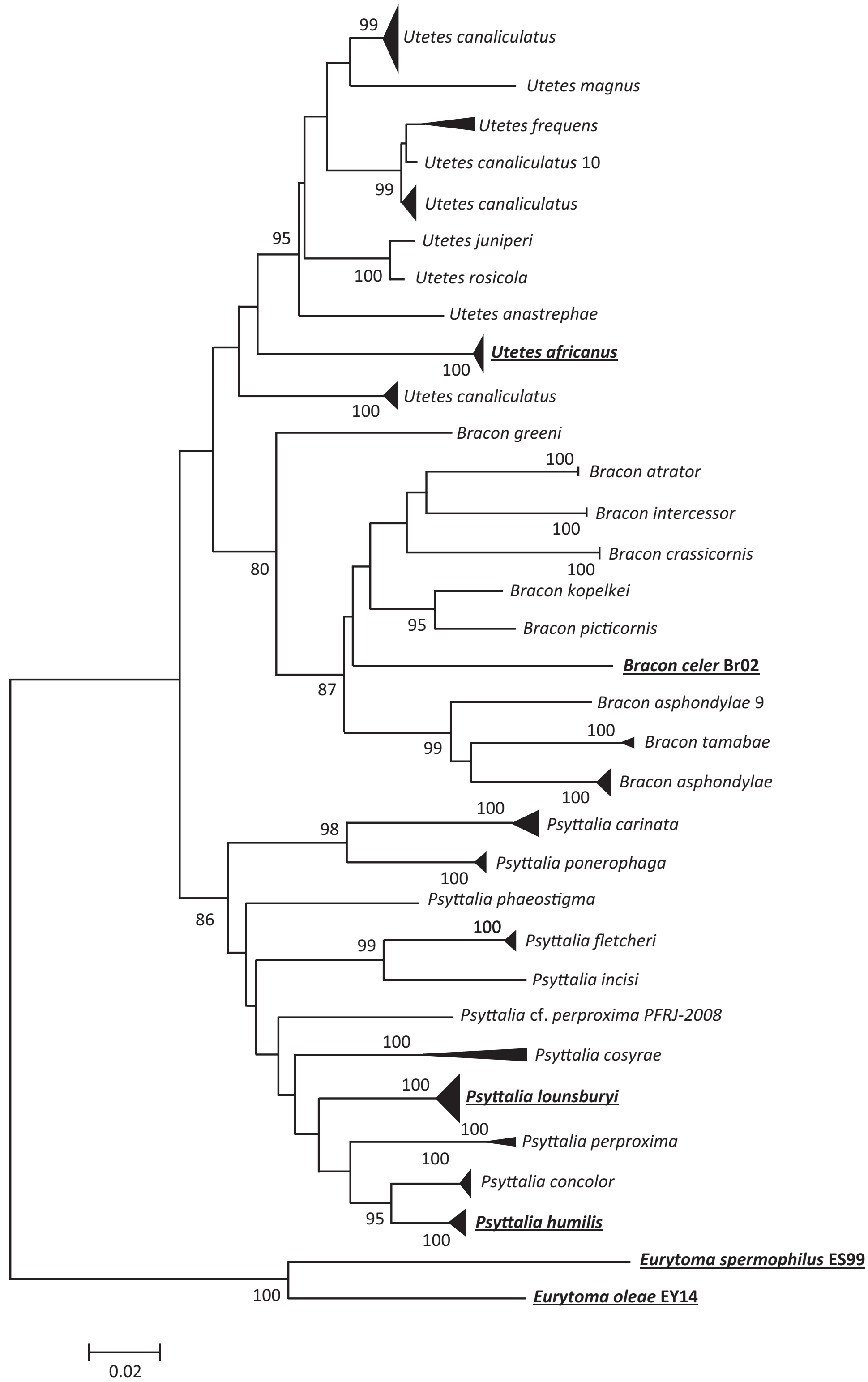
Discussion

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

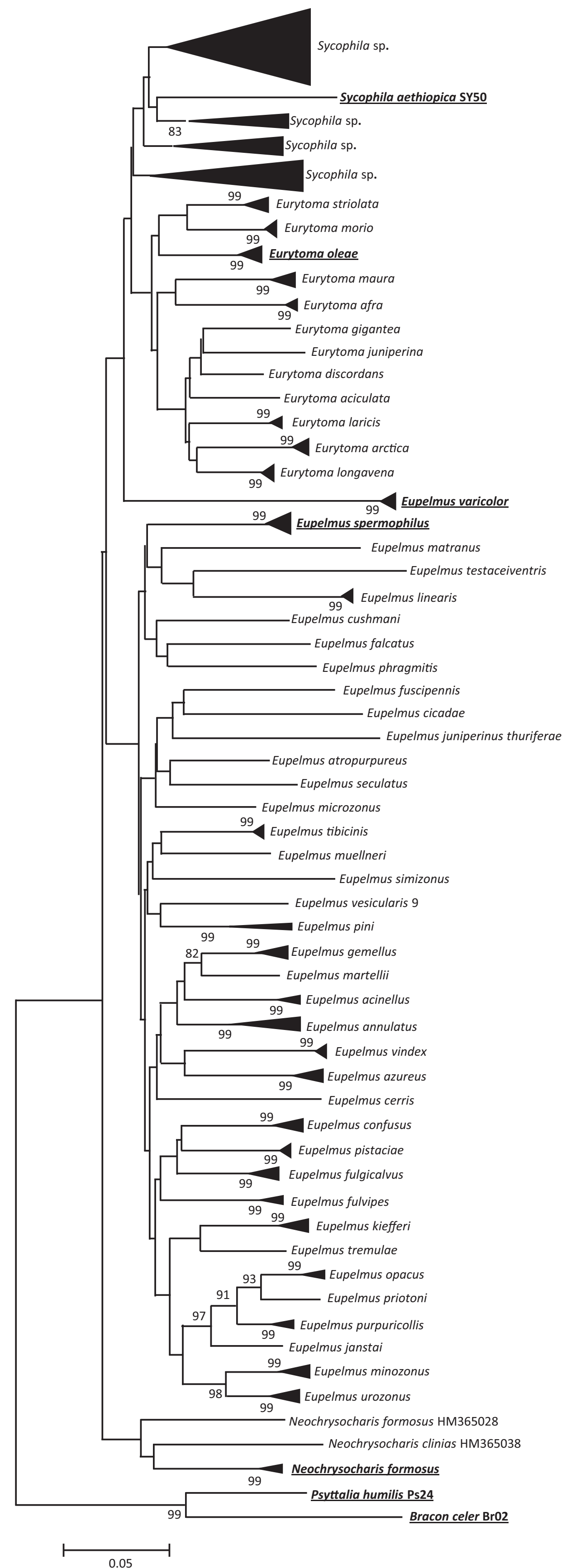
Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

Fig. 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 (1000 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.



Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

Fig. 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 of sites. Values indicate nodal bootstrap support (1000 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.



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.

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 (Hebert et al. 2003, 2004; Ross et al. 2003, 2008; Collins et al. 2012). 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.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

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 branches, 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 and 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 statistical 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%).

Braconidae

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 (Silvestri 1913, 1915; Neuenschwander 1982; Mkize et al. 2008; Daane et al. 2011). 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; Mkize et al. 2008; Rugman-Jones et al. 2009; Daane et al. 2011), and both have been tested as exotic biocontrol agents of *B. oleae* in Europe and California, albeit with limited success (Daane et al. 2008; Borowiec et al. 2012). Previous studies of the genus *Psyttalia* based on *COI* sequences showed monophyly of *Psyttalia* species, including *P. lounsburyi* and *P. humilis* (Cheyppe-Buchmann et al. 2011; Borowiec et al. 2012; Schuler et al. 2016), and phylogenetic reconstruction based on *COI* and 28sD2 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 (Silvestri 1913; Copeland et al. 2004; Mkize et al. 2008; Daane et al. 2011). It has been reported as being more abundant in wild olives than in cultivated olives (Neuenschwander 1982; Mkize et al. 2008; Giacalone 2011; Caleca et al. 2017), 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.

Chalcidoidea

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 (Silvestri 1915; Mkize et al. 2008). In agreement with previous phylogenetic analyses focusing on the *Eupelmus urozonus* species complex (Al Khatib et al. 2014, 2016), 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

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

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 (Fig. S4[2]). 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 (Silvestri 1915; Neuenschwander 1982). 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) (Silvestri 1915; Neuenschwander 1982), 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 and 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 (Neuenschwander 1982; Mkize et al. 2008; Giacalone 2011). 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.

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).

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.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

Acknowledgements

The authors are grateful to numerous olive growers and landowners for allowing the collection of samples on their properties. Emma Cook is acknowledged for assistance in laboratory work. This study was funded by the National Research Foundation (FBIP - Small Grants and Biodiversity Surveys, FBIS150603118644, Grant 98112), the National Research and Technology Fund of South Africa (Reference RTF150428117617; Grant 98590), and Stellenbosch University (Staff Grant, Research Sub-committee B).

References

Al Khatib, F., Fusu, L., Cruaud, A., Gibson, G., Borowiec, N., Rasplus, J.-Y., et al. 2014. An integrative approach to species discrimination in the *Eupelmus urozonus* complex (Hymenoptera, Eupelmidae), with the description of 11 new species from the Western Palaearctic. Syst. Entomol. **39**(4): 806–862. doi:10.1111/syen.12089.

Al Khatib, F., Cruaud, A., Fusu, L., Genson, G., Rasplus, J.-Y., Ris, N., et al. 2016. Multilocus phylogeny and ecological differentiation of the "*Eupelmus urozonus* species group" (Hymenoptera, Eupelmidae) in the West-Palaearctic. BMC Evol. Biol. **16**(1): 13. doi:10.1186/s12862-015-0571-2. PMID:26781031.

Ball, S.L., Hebert, P.D.N., Burian, S.K., and Webb, J.M. 2005. Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. J. N. Am. Benthol. Soc. **24**(3): 508–524. doi:10.1899/04-142.1.

Borowiec, N., Groussier-Bout, G., Vercken, E., Thaon, M., Auguste-Maros, A., Warot-Fricaux, S., et al. 2012. Diversity and geographic distribution of the indigenous and exotic parasitoids of the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), in Southern France. IOBC-WPRS Bull. **79**(i): 71–78.

Bouček, Z., Watsham, A., and Wiebes, J.T. 1981. The fig wasp fauna of the receptacles of *Ficus thonningii* (Hymenoptera, Chalcidoidea). Tijdschr. Entomol. **124**(5): 149–233.

Burks, R.A., Heraty, J.M., Gebiola, M., and Hansson, C. 2011. Combined molecular and morphological phylogeny of Eulophidae (Hymenoptera: Chalcidoidea), with focus on the subfamily Entedoninae. Cladistics, **27**(6): 581–605. doi:10.1111/j.1096-0031.2011.00358.x.

Caleca, V., Giacalone, C., and Allsopp, E. 2015. Olive fruit fly: a threat to the South African olive industry? Technology, South African Olive Industry Association. August/September, pp. 71–72.

Caleca, V., Giacalone, C., Maltese, M., and Tortorici, F. 2017. Contenimento naturale di *Bactrocera oleae* (Rossi): Clima o parassitoidi? Confronto tra Western Cape (Sud Africa) e Sicilia. Atti Acc. Naz. Ital. Entomol. **64**: 99–105.

Chen, Y., Xiao, H., Fu, J., and Huang, D.-W. 2004. A molecular phylogeny of eurytomid wasps inferred from DNA sequence data of 28S, 18S, 16S, and COI genes. Mol. Phyl. Evol. **31**(1): 300–307. doi:10.1016/S1055-7903(03)00282-3. PMID:15019626.

Cheyppe-Buchmann, S., Bon, M.C., Warot, S., Jones, W., Malausa, T., Fauverge, X., and Ris, N. 2011. Molecular characterization of *Psyttalia lounsburyi*, a candidate biocontrol agent of the olive fruit fly, and its *Wolbachia* symbionts as a prerequisite for future intraspecific hybridization. BioControl, **56**(5): 713–724. doi:10.1007/s10526-011-9346-x.

Chien, C.C., and Ku, S.C. 2001. Appearance and life history of *Neochrysocharis formosus* (Hymenoptera: Eulophidae). Formosan Entomol. **21**(4): 383–393.

Collins, R.A., Boykin, L., Cruickshank, R.H., and Armstrong, K.F. 2012. Barcoding's next top model: an evaluation of nucleotide substitution models for specimen identification. Methods Ecol. Evol. **3**(3): 457–465. doi:10.1111/j.2041-210X.2011.00176.x.

Copeland, R., White, I.M., Okumu, M., Machera, P., and Wharton, R.A. 2004. Insects associated with fruits of the Oleaceae (Asteridae, Lamiales) in Kenya, with special reference to the Tephritidae (Diptera). *In* D. Elmo Hardy Memorial Volume. Contribution to the Systematics and Evolution of Diptera. *Edited by* N.L. Evenhuis and K.Y. Kaneshiro. Bishop Mus. Bull. Entomol. **12**: 135–164.

Costa, C. 1998. Olive production in South Africa: a handbook for olive growers. ARC - Institute for Tropical and Subtropical Crops, Nelspruit, South Africa. [ISBN-10: 1868491005.]

Daane, K.M., and Johnson, M.W. 2010. Olive fruit fly: managing an ancient pest in modern times. Annu. Rev. Entomol. **55**: 151–169. doi:10.1146/annurev.ento.54.110807.090553. PMID:19961328.

Daane, K.M., Sime, K.R., Johnson, M.W., Walton, V.M., Nadel, H., Johnson, M.W., et al. 2008. *Psyttalia lounsburyi* (Hymenoptera: Braconidae), potential biological control agent for the olive fruit fly in California. Biol. Control. **44**(1): 79–89. doi:10.1016/j.biocontrol.2007.08.010.

Daane, K.M., Johnson, M.W., Pickett, C.H., Simen, K., Wang, X., Nadel, H., et al. 2011. Biological controls investigated to aid management of olive fruit fly in California. Calif. Agric. **65**(1): 21–28. doi:10.3733/ca.v065n01p21.

Dupuis, J.R., Roe, A.D., and Sperling, F.A. 2012. Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. Mol. Ecol. **21**: 4422–4436. doi:10.1111/j.1365-294X.2012.05642.x. PMID:22891635.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. **3**(5): 294–299. PMID:7881515.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

Gates, M., and Delvare, G. 2008. A new species of *Eurytoma* (Hymenoptera: Eurytomidae) attacking *Quadrastichus* spp. (Hymenoptera: Eulophidae) galling *Erythrina* spp. (Fabaceae), with a summary of African *Eurytoma* biology and species checklist. Zootaxa, **1751**: 1–24.

Giacalone, C. 2011. Il controllo di *Bactrocera oleae* (Rossi) e di altri carpofagi negli oliveti biologici in Sicilia e Sud Africa. Ph.D. thesis, Entomology Department, Palermo University, Palermo, Italy. Available from https://iris.unipa.it/retrieve/handle/10447/95135/121949/merged_document_3.pdf.

Gibson, G.A.P., and Fusu, L. 2016. Revision of the Palaearctic species of *Eupelmus* (*Eupelmus*) Dalman (Hymenoptera: Chalcidoidea: Eupelmidae). Zootaxa, **4081**(1): 1–331. doi:10.11646/zootaxa.4081.1.1.

Green, P.S. 2002. A revision of *Olea* L. (*Oleaceae*). Kew Bull. **57**: 91–140. doi:10.2307/4110824.

Hamerlinck, G., Hulbert, D., Hood, G.R., Smith, J.J., and Forbes, A.A. 2016. Histories of host shifts and cospeciation among free-living parasitoids of *Rhagoletis* flies. J. Evol. Biol. **29**(9): 1766–1779. doi:10.1111/jeb.12909. PMID:27234648.

Hebert, P.D., and Gregory, T.R. 2005. The promise of DNA barcoding for taxonomy. Syst. Biol. **54**(5): 852–859. doi:10.1080/10635150500354886. PMID:16243770.

Hebert, P.D., Cywinska, A., Ball, S.L., and deWaard, J.R. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. B Biol. Sci. **270**(1512): 313–321. doi:10.1098/rspb.2002.2218. PMID:12614582.

Hebert, P.D.N., Penton, E.H., Burns, J.M., Janzen, D.H., and Hallwachs, W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*. Proc. Natl. Acad. Sci. U.S.A. **101**(41): 14812–14817. doi:10.1073/pnas.0406166101. PMID:15465915.

Hoelmer, K.A., Kirk, A.A., Pickett, C.H., Daane, K.M., and Johnson, M.W. 2011. Prospects for improving biological control of olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), with introduced parasitoids (Hymenoptera). Biocontrol Sci. Technol. **21**(9): 1005–1025. doi:10.1080/09583157.2011.594951.

Hood, G.R., Forbes, A.A., Powell, T.H., Egan, S.P., Hamerlinck, G., Smith, J.J., and Feder, J.L. 2015. Sequential divergence and the multiplicative origin of community diversity. Proc. Natl. Acad. Sci. U.S.A. **112**(44): E5980–E5989. doi:10.1073/pnas.1424717112. PMID:26499247.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, **28**(12): 1647–1649. doi:10.1093/bioinformatics/bts199. PMID:22543367.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**(2): 111–120. doi:10.1007/BF01731581. PMID:7463489.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. **33**(7): 1870–1874. doi:10.1093/molbev/msw054. PMID:27004904.

Leite, L.A.R. 2012. Mitochondrial pseudogenes in insect DNA barcoding: differing points of view on the same issue. Biota Neotrop. **12**(3): 301–308. doi:10.1590/S1676-06032012000300029.

Lotfalizadeh, H., Delvare, G., and Rasplus, J.-Y. 2007. Phylogenetic analysis of Eurytominae (Chalcidoidea: Eurytomidae) based on morphological characters. Z. J. Linn. Soc. **151**: 441–510. doi:10.1111/j.1096-3642.2007.00308.x.

Matsuo, K., Uechi, N., Tokuda, M., Maeto, K., and Yukawa, J. 2016. Host range of braconid species (Hymenoptera: Braconidae) that attack Asphondyliini (Diptera: Cecidomyiidae) in Japan. Entomol. Sci. **19**(1): 3–8. doi:10.1111/ens.12167.

Miller, M.A., Pfeiffer, W., and Schwartz, T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *In* Proceedings of the Gateway Computing Environments Workshop, 14 Nov. 2010, New Orleans, La. pp. 1–8. doi:10.1109/GCE.2010.5676129.

Min, X.J., and Hickey, D.A. 2007. Assessing the effect of varying sequence length on DNA barcoding of fungi. Mol. Ecol. Notes, **7**(3): 365–373. doi:10.1111/j.1471-8286.2007.01698.x. PMID:18784789.

Mkize, N., Hoelmer, K.A., and Villet, M.H. 2008. A survey of fruit-feeding insects and their parasitoids occurring on wild olives, *Olea europaea* ssp. *cuspidata*, in the Eastern Cape of South Africa. Biocontrol Sci. Technol. **18**(10): 991–1004. doi:10.1080/09583150802450154.

Munro, H.K. 1926. Biological notes on South-African Trypaneidae (Trypaneidae: fruit flies). II. Entomol. Mem. S. Afr. Dep. Agric. **5**: 17–40.

Munro, H.K. 1984. A taxonomic treatise on the Dacidae (Tephritidae, Diptera) of Africa. Entomol. Mem. S. Afr. Dep. Agric. **61**: I–IX, pp. 1–313.

Nardi, F., Carapelli, A., Dallai, R., Roderick, G.K., and Frati, F. 2005. Population structure and colonization history of the olive fly, *Bactrocera oleae* (Diptera, Tephritidae). Mol. Ecol. **14**(9): 2729–2738. doi:10.1111/j.1365-294X.2005.02610.x. PMID:16029474.

Nardi, F., Carapelli, A., Boore, J.L., Roderick, G.K., Dallai, R., and Frati, F. 2010. Domestication of olive fly through a multi-regional host shift to cultivated olives: comparative dating using complete mitochondrial genomes. Mol. Phylogenet. Evol. **57**(2): 678–686. doi:10.1016/j.ympev.2010.08.008. PMID:20723608.

Neuenschwander, P. 1982. Searching parasitoids of *Dacus oleae* (Gmel.) (Dipt., Tephritidae) in South Africa. Zeit. Ang. Entomol. **94**(1): 509–522. doi:10.1111/j.1439-0418.1982.tb02598.x.

Nieves-Aldrey, J.L., Hernández Nieves, M., and Gómez, J.F. 2007. A new afrotropical species of *Ormyrus* Westwood, 1832 (Hymenoptera, Chalcidoidea, Ormyridae). Graellsia, **63**(1): 53–60. doi:10.3989/graellsia.2007.v63.i1.80.

Ramezani, S., Blibech, I., Trindade-Rei, F., van Asch, B., and Teixeira da Costa, L. 2015. *Bactrocera oleae* (Diptera: Tephritidae) in Iran: an invasion from the Middle West. Eur. J. Entomol. **112**(4): 713–721. doi:10.14411/eje.2015.097.

Ratnasingham, S., and Hebert, P.D. 2007. Bold: The Barcode of Life Data System (http://www.barcodinglife.org). Mol. Ecol. Notes, **7**(3): 355–364. doi:10.1111/j.1471-8286.2007.01678.x. PMID:18784790.

Rice, R.E., Phillips, P.A., Stewart-Leslie, J., and Sibbett, G.S. 2003. Olive fruit fly populations measured in Central and Southern California. Calif. Agric. **57**(4): 122–127. doi:10.3733/ca.v057n04p122.

Ross, H.A., Lento, G.M., Dalebout, M.L., Goode, M., Ewing, G., McLaren, P., et al. 2003. DNA Surveillance: Web-based molecular identification of whales, dolphins, and porpoises. J. Hered. **94**(2): 111–114. doi:10.1093/jhered/esg027. PMID:12721222.

Ross, H.A., Murugan, S., and Li, W.L.S. 2008. Testing the reliability of genetic methods of species identification via simulation. Syst. Biol. **57**(2): 216–230. doi:10.1080/10635150802032990. PMID:18398767.

Rowley, D.L., Coddington, J.A., Gates, M.W., Norrbom, A.L., Ochoa, R.A., Vandenberg, N.J., and Greenstone, M.H. 2007. Vouchering DNA-barcoded specimens: test of a nondestructive extraction protocol for terrestrial arthropods. Mol. Ecol. Notes, **7**(6): 915–924. doi:10.1111/j.1471-8286.2007.01905.x.

Rubio, De, Casas, R., Besnard, G., Schönswetter, P., Balaguer, L., and Vargas, P. 2006. Extensive gene flow blurs phylogeographic but not phylogenetic signal in *Olea europaea* L. Theor. Appl. Genet. **113**(4): 575–583. doi:10.1007/s00122-006-0306-2. PMID:16835765.

Rugman-Jones, P.F., Wharton, R., van Noort, T., and Stouthamer, R.

2009. Molecular differentiation of the *Psyttalia concolor* (Szépligeti) species complex (Hymenoptera: Braconidae) associated with olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), in Africa. Biol. Control, **49**(1): 17–26. doi:10.1016/j.biocontrol.2008.12.005.

Sambrook, J., Fritsch, E., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schuler, H., Kern, P., Arthofer, W., Vogt, H., Fischer, M., Stauffer, C., and Riegler, M. 2016. *Wolbachia* in parasitoids attacking native European and introduced Eastern cherry fruit flies in Europe. Environ. Entomol. **45**(6): 1424–1431. doi:10.1093/ee/nvw137. PMID:28028089.

Silvestri, F. 1913. Viaggio in Africa per cercare parassiti di mosche dei frutti. Boll. Lab. Zoo. Gen. Agr. R. Scu. Sup. d'Agric. Portici, **8**: 1–164.

Silvestri, F. 1915. Contributo alla conoscenza degli insetti dell' olivo dell' Eritrea e dell' Africa meridionale. Boll. Lab. Zoo. Gen. Agr. Portici, **9**: 240–334.

Silvestri, F. 1939. La lotta biologica contro le mosche dei frutti della famiglia Trypetidae. *In* Proceedings of VII International Congress of Entomology, 15–20 August, Berlin, Germany, Vol. 4. pp. 2396–2418.

Smith, M.A., Bertrand, C., Crosby, K., Eveleigh, E.S., Fernandez-Triana, J., Fisher, B.L., et al. 2012. *Wolbachia* and DNA barcoding insects: patterns, potential, and problems. PLoS ONE, **7**(5): e36514. doi:10.1371/journal.pone.0036514. PMID:22567162.

Stamatakis, A. 2006. Phylogenetic models of rate heterogeneity: a high performance computing perspective. *In* Proceedings of the 20th IEEE International Parallel & Distributed Processing Symposium (IPDPS2006), IEEE Computer Society Press, Washington, pp. 278–286. doi:10.1109/IPDPS.2006.1639535.

Stamatakis, A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics, **30**(9): 1312–1313. doi:10.1093/bioinformatics/btu033. PMID:24451623.

Wharton, R., and Yoder, M. Parasitoids of fruit-infesting Tephritidae. [Online.] Available from http://paroffit.org [accessed 10 March. 2017].

Zohary, D. 1994. The wild genetic resources of the cultivated olive. Acta Hortic. **356**: 62–65. doi:10.17660/ActaHortic.1994.356.12.

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.

This article has been cited by:

1. Sarah J. Adamowicz, James S. Boatwright, Frédéric Chain, Brian L. Fisher, Ian D. Hogg, Florian Leese, Darío A. Lijtmaer, Monica Mwale, Amanda M. Naaum, Xavier Pochon, Dirk Steinke, John-James Wilson, Susanna Wood, Jianping Xu, Sen Xu, Xin Zhou, Michelle van der Bank. 2019. Trends in DNA barcoding and metabarcoding. *Genome* **62**:3, v-viii. [Citation] [Full Text] [PDF] [PDF Plus]

Genome Downloaded from www.nrcresearchpress.com by STELLENBOSCH UNIVERSITY on 04/25/19
For personal use only.