Molecular differentiation of the *Psyttalia concolor* (Szépligeti) species complex (Hymenoptera: Braconidae) associated with olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), in Africa

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**Abstract**

The genus *Psyttalia* (Braconidae: Opiinae) contains several species being used or considered for use in the biological control of various fruit-infesting tephritid pests, most notably olive fly, *Bactrocera oleae* and Medfly, *Ceratitis capitata*. There is continued interest in obtaining more effective tephritid parasitoids, and much attention has focused on one particular group of closely related species from subsaharan Africa, the *P. concolor* species complex. However, considerable confusion surrounds the identity of members of this complex because they are difficult to differentiate morphologically. We provide information on nuclear and mitochondrial DNA markers that may be used for separation of various populations. Phylogenetic analyses using sequence data from 28sD2 and COI gene regions illustrate relationships among 10 identifiable groups of *Psyttalia* populations, and we subsequently discuss (and assign) available species names for these populations. The name *Psyttalia humilis* (Silvestri) is available for subsaharan populations that are morphologically similar to *Psyttalia concolor*, the name applicable to populations from the Mediterranean region. Our findings are further discussed in relation to the potential use of several populations in biological control efforts against olive fly in California.

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1. Introduction

The braconid genus *Psyttalia* Walker (treated until recently as a subgenus or synonym of *Opisus* Wesmael) contains 87 nominal species, all endemic to the Old World (Fischer, 1972, 1987; Wharton, 1987; Kimani-Njogu et al., 2001; Yu et al., 2005). Hosts are known for almost half of the currently recognized species (Clausen et al., 1965; Fischer, 1971; Wharton, 1997; Wharton et al., 1999; Chinajarjawong et al., 2000) and they are all solitary, koinobiont endoparasitoids of Tephritidae, developing inside the immature stages of the fly and killing it in the process. As such, *Psyttalia* species have attracted much interest for use as biological control agents (Silvestri, 1913, 1916; Clausen, 1978; Gilstrap and Hart, 1987; Wharton, 1989), and one species, *Psyttalia concolor* (Szépligeti), is widely used in the Mediterranean region for augmentative control of the olive fly, *Bactrocera oleae* (Rossi) (Raspi, 1995).

The correct identification of a natural enemy is fundamental to its effective use as a biological control agent. *Psyttalia concolor* is a member of a complex of closely related species from Africa, that have been used extensively in both classical and augmentative biological control programs directed against tephritid pests (Clausen et al., 1965; Clausen, 1978; Gilstrap and Hart, 1987; Wharton, 1989). In part because of its geographical distribution, there is little doubt about the identity of the mass-reared *Psyttalia* used for olive fly control around the Mediterranean. However, because they cannot be readily separated based on morphology, considerable confusion surrounds the identity of other members of the *P. concolor* species complex attacking various tephritids in subsaharan Africa (Fischer, 1958, 1963, 1971, 1972; Wharton and Gilstrap, 1983; Wharton, 1987, 1988, 1997; Kimani-Njogu et al., 2001). Members of the *P. concolor* species complex that have proven challenging to differentiate, and whose names have been variously applied in biological control programs directed against tephritid pests, include *P. humilis* (Silvestri), *P. perproxima* (Silvestri), *P. phaeostigma* (Wilkinson), *P. ponerophaga* (Silvestri), and *P. dacida* (Silvestri). Additionally, *P. cosyrae* (Wilkinson) and *P. lounsburyi* (Silvestri) have traditionally been distinguished by a somewhat longer ovispositor and darker coloration, respectively, but are otherwise morphologically very similar (Wharton and Gilstrap, 1983; Kimani-Njogu et al., 2001).

*Psyttalia concolor* was originally described in 1910 from material reared from Tunisian olives (Szépligeti, 1910), and shortly thereafter, was introduced to olive-growing regions of Italy and France. Following the development of an efficient mass-rearing technique in the 1950s, augmentative releases of *P. concolor*...
against olive fly began in these regions and continue to this day. A second species, *P. humilis*, was described by Silvestri (1913), based on specimens he reared from pears infested by Medfly, *Ceratitis capitata* (Wiedemann), in the vicinity of Cape Town, South Africa. *Psyttalia humilis* was quickly and successfully established in Hawaii, and brought about significant reductions of Medfly in coffee crops (Back and Pemberton, 1918). Following this success, *P. humilis* was redistributed to several other countries over the next two decades (Clausen 1978; Wharton, 1989). However, these later introductions never established and specimens recognized as *P. humilis* have not been recovered from Hawaii or encountered elsewhere since the mid 1930s (Willard and Mason, 1937; Wharton, 1989). Morphologically, *P. humilis* is virtually indistinguishable from *P. concolor*, and subsequent to its use in biological control in the first half of the 20th century, *P. humilis* has sometimes been treated as a junior synonym of *P. concolor* (Fischer, 1958, 1963, 1971, 1972; Wharton and Gilstrap, 1983; Wharton et al., 2000).

Several other nominal species have been proposed on the basis of subtle differences in such features as the length of the ovipositor and the size of the eye (Silvestri, 1913; Wilkinson, 1927; Wharton and Gilstrap, 1983). Wilkinson (1927) described *P. cosyrae* from two specimens reared from *Ceratitis cosyra* (Walker) in Tanzania, that had noticeably longer ovipositors than a second species he described as *P. phaeostigma* from unknown hosts collected in Durban, South Africa. Unfortunately, Wilkinson’s sole female specimen of *P. phaeostigma* was poorly preserved, with the ovipositor retracted within the abdomen, and comparison of Wilkinson’s type material suggests that *P. cosyrae* and *P. phaeostigma* may have equally long ovipositors (Wharton and Gilstrap, 1983). The problem is further complicated by the fact that Silvestri (1913) described *P. perproxima* and *P. perproxima modestior* from tropical West Africa based on specimens reared from a variety of fruits, some containing *C. cosyra* (reported as *C. giffardi* Bezzi) and others containing the cucurbit-infesting *Dacus ciliatus* Loew (reported as *D. brevistylus* Bezzi). It is possible that at least some of Silvestri’s material (that reared from *C. cosyra*) is the same as Wilkinson’s *P. cosyrae*, while some of the other specimens (reared from *D. ciliatus*) are the same as Wilkinson’s *P. phaeostigma*. Furthermore, the reliability of morphological characters has been questioned since the size and color of the adult wasps appear to be correlated with the size and identity of their hosts (Kimani-Njogu et al., 2001; Billah et al., 2005). Thus, it is also possible that all four names apply to the same species (Wharton and Gilstrap, 1983).

There has been repeated interest in obtaining more effective tephritid parasitoids than those currently available (Mensing, 1996; Sivinski, 1996), and Africa is a logical focus of attention because such widespread pests as Medfly and olive fly are indigenous there (Gilstrap and Hart, 1987; Wharton, 1989; Headrick and Goe- den, 1996; De Meyer et al., 2004; Copeland et al., 2004). One of the dominant parasitoids reared from tephritids infesting coffee (*Coffea arabica* L.) fruits in central Kenya appears to be morphologically identical to, and is reproductively compatible with, populations of *P. concolor* from Italy (Kimani-Njogu et al., 2001; Billah et al., 2008). Individuals from Kenyan and Italian populations are also capable of successfully developing on both Medfly and olive fly. Here, we examine levels of genetic differentiation among populations of *Psyttalia* from localities in northern, central (Kenya), and southern Africa, and compare these with populations of *P. concolor* from Italy, *P. loubsburyi* (Silvestri) from Africa, and *P. ponerophaga* from Pakistan. This work seeks to add “molecular” resolution to the morphological identification of members of the *P. concolor* species complex, especially those being considered for control of olive fly in California (e.g. Sime et al., 2006c, 2007). Furthermore, this work identifies population-level differences that might be used as genetic markers in biological control. The success of biological control programs may rely heavily on the introduction of an optimal pop-

ulation (or cryptic species) (DeBach and Rosen, 1991). The *P. concolor* species complex offers an opportunity to identify such optimal populations, because of the potential for releasing individuals from a variety of source populations (perhaps representing different climatic regimes) against various target pests; in addition to olive fly and Medfly, members of the *P. concolor* species complex also attack *Ceratitis cosyra*, a serious pest of mango (*Mangifera indica* L.) in Africa. Following simultaneous releases, characterization of genetic markers will make it possible to identify which (if any) source populations (or cryptic species) become established.

2. Materials and methods

2.1. Origin of material

The focal material for this study came from laboratory cultures and field-collected fruits (summarized in Table 1). As reflected in the text below, all populations were assigned an alpha-code (column 5, Table 1), since species-level identification, one of the objectives of this study, was often problematic. Laboratory cultures of the Italian *Psyttalia concolor*, reared on Medfly, were obtained from a mass-rearing facility in Pisa, Italy (population A), established primarily for augmentative releases against olive fly (Raspi and Loni, 1994). Wasps originating from the same source but previously (1998–2000) established at the International Centre for Insect Physiology and Ecology (ICIPE) in Nairobi, Kenya, and at the University of Hawaii (population B), and subsequently at the University of California, Berkeley (population C) (Sime et al., 2006c), were obtained for comparison. Laboratory cultures of a morphologically similar *Psyttalia* from Kenya (populations K and L, ultimately identified as *P. humilis*, see Sections 3 and 4) were also established at ICIPE from fruits sampled in 1998 from two different coffee (*Coffea arabica* L., Rubiaceae) plantations (Ruiru and Rurima) in the central highlands of Kenya near Nairobi (Wharton et al., 2000; Kimani-Njogu et al., 2001). These cultures were subsequently used to establish lab colonies of the native Kenyan *Psyttalia* in Guatemala, Hawaii, and California (populations M and N) using either Medfly or olive fly as hosts. Each of these cultures was sampled in the present study.

Specimens similar in appearance to *P. concolor* and ultimately determined to represent several different species, were also reared from several wild host plants in Kenya, and from wild olives in Morocco (population D), the Canary Islands (population E), Namibia (population O), and South Africa (population P). Procedures for handling field-collected fruits are outlined in Wharton et al. (2000) and Copeland et al. (2002). Wasps were directly preserved in 95% ethanol upon emergence from the field-collected fruits. Kenyan samples were obtained in 2000 from two sites along the coast: Arabuko-Sokoke forest (populations J and T) and adjacent areas north of MOMBASA; and, the Shamba Hills region near Muhaka, south of MOMBASA (populations F and S). The Arabuko-Sokoke forest is 120 km NNW of the Shamba Hills region. Fruits yielding *Psyttalia* from these coastal sites included members of the Annonaceae (*Letttowianthus stellatus* Diels), Flagellariaceae (*Flagellaria guineensis* Schumach), and Rubiaceae (*Chazalia abrupta* var. *parvifolia* Verdc., *Psychotria lauracea* (K. Schum.) E.M.A. Petit, and *P. capensis* (Eckl.) Vatke). In addition to Medfly, the following tephritid species of the subtribe Ceratitidina were reared from these fruit samples (Table 1): *Ceratitis pinax* (Munro), *C. rosa* (Karsch), *Trihi-thrum nigerrimum* (Bezzi), *T. senex* (Munro), and *T. teres* (Munro). *Psyttalia* specimens were also collected in 2005 from an *Acokanthera* sp. (Apocynaceae) infested with Medfly and Ceratitis *simi* (Munro) from the Ololua Forest, 10 miles SW of Nairobi in central Kenya (population H) and with a sweep net on coffee at Tafo, Gha-

na (population U).
Samples from several other populations of the *Psyttalia concolor* complex were obtained to assess the utility of the three gene regions (see below) for differentiating both species and populations (Table 1). Two species with distinctly longer ovipositors than *P. concolor* were established as lab cultures at ICIE (Mohamed et al., 2003). Specimens tentatively identified as *Psyttalia cosyrae* (population G) were originally collected from Cae-loptic larvae from these sources. *Psyttalia lounsburyi*, a dark species with a short ovipositor, was obtained from wild olives (*Olea* spp., *Olea europaea* L.ssp. *cuspida*data) [Wall ex G. Don] Cif., in Burguret Forest on the slopes of Mt. Kenya (Copeland et al., 2004) (population Q), and near Stellenbosch, in South Africa (population R). Our study was completed by the inclusion of *Psyttalia ponerophaga* (Silvestri) reared from olives in Cherat, Pakistan (population W). 'Host' flies reared from olives from Morocco, Namibia, Pakistan, and the Canary Islands were all olive fly, *Bactrocera oleae*. However, in South Africa, olives were infested with both olive fly and *Bactrocera biguttula* (Munro), and in Kenya by olive fly and *Bactrocera munroi* (White).

Although all but one (population U) of the *Psyttalia* used in this study were reared from tephritid puparia, it was not possible to determine which species served as host(s) of the parasitoids for field-collected samples where more than one fly species emerged (Table 1). Voucher specimens for plants are deposited at ICIE and the National Museums of Kenya; voucher specimens for host flies are in the Tervuren Museum (Belgium) and The Natural History Museum (London); and for *Psyttalia* in the Texas A&M University (TAMU) Insect Collection.

### 2.2. DNA extraction, amplification and sequencing

Wasps were shipped to the University of California, Riverside in 95% ethanol. Total DNA was isolated from a single hind tibia using the “salting-out” method advocated by Rugman-Jones et al. (2006). Following successful DNA extraction, three separate gene regions were amplified using the polymerase chain reaction (PCR). In separate reactions, a 660 bp (primers removed) section of the cytochrome oxidase c subunit I (COI) gene of mtDNA and a 567 bp section of the 28sD2 region of ribosomal RNA were amplified using the respective primer pairs: LCO 1490 (5'-GGTCAACAATCATATAAAGAGATATTGG-3') paired with HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and 1 min at 72°C; followed by five cycles of 30 s at 94°C; followed by a further 35 cycles of 30 s at 94°C, 1 min 30 s at 45°C, and 1 min at 72°C; followed by a further 35 cycles of 30 s at 94°C, 1 min 30 s at 51°C, and 1 min at 72°C; and a final extension of 5 min at 72°C. Amplification of the 28sD2 region was performed in 50 μL reactions containing 5 μL of DNA template (concentration not determined), 1 × PCR Buffer (containing 2 mM MgSO4; New England Biolabs, Ipswich, MA, USA), 20 μL each dNTP, 2 mM MgCl2, 2.5 μL BSA (NEB), 0.2 μM each primer, and 2 U Taq polymerase (NEB). Amplification was performed in a Mastercycler 5331 (Eppendorf, Hamburg, Germany) programmed for: an initial denaturing step of 2 min at 94°C; followed by five cycles of 30 s at 94°C, 1 min 30 s at 45°C, and 1 min at 72°C; followed by a further 35 cycles of 30 s at 94°C, 1 min 30 s at 51°C, and 1 min at 72°C; and a final extension of 5 min at 72°C. Amplification of the 28sD2 region was performed in 50 μL reactions containing 2 μL of DNA template (concentration not determined), 1 × PCR Buffer (containing 2 mM MgSO4; NEB), 20 μL each dNTP, 0.2 μM each primer, and 1 U Taq polymerase (NEB). The thermocycler was programmed for: an initial denaturing step of 3 min at 94°C; followed by 30 cycles of 45 s at
94 °C, 30 s at 55 °C, and 1 min 30 s at 72 °C; and a final extension of 5 min at 72 °C.

Where possible (see Section 3), a 600-700 bp section of the internal transcribed spacer region 2 (ITS2) was also amplified using the forward primers ITS2 Psyttalia F1 (5'-GATCTAATTCGACGACCAAT-3' van Noort, unpublished data) or ITS2-F (5'-TGGAACTTGCAGCAGATAG-3') paired with the reverse primer ITS2-R (5'-AGCTTCGGCTGCTTCAAGGT-3') (Campbell et al., 1993, 2000). Amplifications were again performed in 25 μL reactions with the same reaction mix as that used for the 28sD2 region. The thermocycler was programmed for: an initial denaturing step of 3 min at 95 °C; followed by 34 cycles of 45 s at 92 °C, 45 s at 59.7 °C, and 45 s at 72 °C; and a final extension of 3 min at 72 °C.

Amplified DNA was cleaned using the Wizard® PCR Prep DNA purification system (Promega, Madison, WI, USA). The COI and 28sD2 regions were direct sequenced in both directions at the University of California Riverside Genomics Institute, Core Instrumentation Facility using an Applied Biosystems 3730 DNA analyzer with a Big-Dye® V3.1 kit (Applied Biosystems, Foster City, CA, USA). The ITS2 was also sequenced but required cloning into a plasmid vector prior to sequencing (for methods see Rugman-Jones et al., 2006). All sequences were initially aligned using ClustalX version 1.83 (Thompson et al., 1997) and then by eye in BioEdit version 7.0.5.3 (Hall, 1999). Sequences are deposited in GenBank (Benson et al., 2008; for accession numbers see Table 1). Population-groups and simple distance-based relationships between these groups, in the 28sD2 and COI regions (Table 2).

Restriction digests of the COI and 28sD2 regions were direct sequenced in both directions at the University of California Riverside Genomics Institute, Core Instrumentation Facility using an Applied Biosystems 3730 DNA analyzer with a Big-Dye® V3.1 kit (Applied Biosystems, Foster City, CA, USA). The ITS2 was also sequenced but required cloning into a plasmid vector prior to sequencing (for methods see Rugman-Jones et al., 2006). All sequences were initially aligned using ClustalX version 1.83 (Thompson et al., 1997) and then by eye in BioEdit version 7.0.5.3 (Hall, 1999). Sequences are deposited in GenBank (Benson et al., 2008; for accession numbers see Table 1). Population-groups and simple distance-based relationships between these groups, in the 28sD2 and COI regions (Table 2). COI sequences were translated at www.ebi.ac.uk/emboss/translate/ and the number of non-synonymous changes within each group was calculated, again using DnaSP.

2.3. Restriction profiles

Diagnostic restriction sites in the COI sequences were sought using the “Restriction Mapping” feature in BioEdit. Restriction digests of the COI region were performed in 10 μL reactions containing 6 U of the restriction enzyme BclI, SspI, ApoI, or NsiI (NEB), 1X its accompanying buffer, and an appropriate amount of unpurified PCR product (7.8–8.6 μL). Reactions containing BclI were incubated for 2 h at 50 °C with no subsequent inactivation (BclI is not inactivated by heating). Reactions containing Apol were similarly incubated at 50 °C but this was followed by a 20 min inactivation step at 80 °C. Those containing Spsl and Nsil were incubated for 2 h at 37 °C and then inactivated with 20 min at 65 and 80 °C, respectively. Restriction products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

2.4. Phylogenetic analysis

The 28sD2 and COI sequences were concatenated and an aligned matrix of 1227 bp was assembled for 31 haplotypes, including two outgroups representing different genera from the same subfamily of Braconidae (Sternaulopius bisternaulicus Fischer and an undescribed species of Utesis, both reared from fruit-infesting tephritids in Kenya). No gaps were necessary in either the 28sD2 or COI partition. The 28sD2 partition was comprised of 567 bases; COI included 660 bases, starting at codon position 3 and including no stop codons.

A Branch and Bound parsimony (BBP) analysis of the combined COI and 28sD2 data was conducted using PAUP 4.0 b10 (Swofford, 2003). A Maximum likelihood (ML) analysis was executed in RAxML 7.0 (Stamatakis et al., 2005; Stamatakis, 2008) as run on the CIPRES web portal (http://www.phylo.org/sub_sections/portal/) with a partitioned nucleotide model for COI and 28sD2. Branch support for each analysis was estimated by nonparametric bootstrapping with 1000 replicates.

3. Results

3.1. Genetic diversity

The three gene regions amplified with varying success. The COI and 28sD2 primer sets consistently yielded reliable amplifications. The concatenated alignment of the 28sD2 and COI sequences confirmed the existence of 10 groups (Fig. 1) and subsequent analyses were performed on these groups, using the species names tentatively assigned in Fig. 1 and Table 1 (see Section 4). Amplification and sequencing of the ITS2 region was more problematic. Two different forward primers were required to amplify the region across all populations (see methods), but even then, it was impossible to get a single “clean” PCR product (Rugman-Jones, unpublished data). Sequencing of the ITS2 region for a restricted number of specimens was facilitated by cloning the fragment into a plasmid vector and alignment of these sequences appeared to define the same groups as did the 28sD2

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>mtDNA (28sD2)</th>
<th>mtDNA (COI)</th>
<th>mtDNA (combined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. concolor (10/10)*</td>
<td>0.2/1.3</td>
<td>0.0/0.0</td>
<td>0.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. humilis (12/10)</td>
<td>1.1/31.4</td>
<td>0.0/0.0</td>
<td>0.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. cosyrae (3/4)</td>
<td>1.1/77.8</td>
<td>0.0/0.0</td>
<td>0.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. lounsburyi (Benin)</td>
<td>1.1/71.0</td>
<td>0.0/0.0</td>
<td>0.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. cosyrae (2/2) (Kenya)</td>
<td>1.1/71.0</td>
<td>0.0/0.0</td>
<td>0.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. lounsburyi (10/8)</td>
<td>2.1/58.0</td>
<td>1.0/0.0</td>
<td>1.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. n. sp. (2/2)</td>
<td>2.1/76.3</td>
<td>1.0/0.0</td>
<td>1.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. perproxima (6/7)</td>
<td>3.9/55.0</td>
<td>2.8/54.8</td>
<td>2.8/54.8</td>
<td></td>
</tr>
<tr>
<td>P. cf. perproxima (3/3)</td>
<td>2.1/68.5</td>
<td>1.0/0.0</td>
<td>1.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. phaeostigma (2/2)</td>
<td>3.1/68.2</td>
<td>2.0/0.0</td>
<td>2.0/0.0</td>
<td></td>
</tr>
<tr>
<td>P. ponerophaga (4/5)</td>
<td>5.1/74.1</td>
<td>4.0/0.0</td>
<td>4.0/0.0</td>
<td></td>
</tr>
</tbody>
</table>

* n. 28sD2/COI, COI.
and COI regions (see GenBank Accession Nos. EU761046–EU761074). However, given the problems we encountered in trying to amplify and sequence this region, and as a result, the incomplete nature of our dataset, further analysis of the ITS2 data was not performed.

Across groups, the 28sD2 region was highly conserved with only 0.9% difference (or a mean of 5.1 nucleotide substitutions over 567 bp) in the most divergent groups, *P. ponerophaga* and *P. concolor* (Table 2). Indeed over the 10 groups the number of segregating (polymorphic) sites was limited to 10 (from 567 bp) and there were only 10 haplotypes, one of which was shared by three groups; the two populations of *P. cosyrae* (from Kenya and Benin) and *P. humilis* (Table 2). As expected, intra-group sequence polymorphism in the 28sD2 region was also very low, with 8 of 10 groups being monomorphic and only *P. perproxima* and *P. concolor* having a second haplotype (Table 2). Across groups, the COI region was more divergent with 4.8–13.9% sequence divergence (or a mean of 31.4–91.5 nucleotide substitutions over 660 bp) between groups, 183 segregating sites (from 660 bp) and 29 haplotypes. The most divergent groups were the populations of *P. cosyrae* from Benin and *P. n. sp.* reared from *Ceratitis rosa* infesting *Lettowianthus stellatus* (Table 2). Intra-group polymorphism in the COI region was also higher, with the number of segregating (polymorphic) sites and number of haplotypes per group ranging from 0 to 10 and 1 to 6, respectively (Table 2). However, within each group, the majority of nucleotide substitutions in the COI region constituted synonymous changes. For example, in the most diverse group based on the nucleotide sequence (*P. humilis*), only 2 of 10 codon changes resulted in a substitution in the coded amino acid sequence. In terms of the amino acid sequence, the *P. concolor* group was the most diverse with three non-synonymous substitutions.

### 3.2. Restriction profiles

Digestion of the COI PCR product with appropriate restriction enzymes yielded diagnostic differences, allowing the construction of a simple key to the 10 groups studied (Table 3).

| (1) Cut with restriction enzyme BclI: | Results in 3 visible bands, smallest >100 bp 2 | Results in 3 visible bands, smallest <100 bp 5 |
| (2) Cut with restriction enzyme SspI: | Results in 4 visible bands  P. lounsburyi | Results in 3 visible bands 3 |
| | Results in 2 visible bands 4 | |
| (3) Largest band 360 bp  P. phaeostigma | Largest band <500 bp  P. concolor |
| (4) Cut with restriction enzyme ApoI: | Results in 2 visible bands, largest ~600 bp P. cosyrae (Kenya) |
| | Results in 2 visible bands, largest ~400 bp P. concolor |
| | Results in 2 visible bands, largest ~300 bp P. n. sp. |
| (5) Cut with restriction enzyme ApoI: | Results in 2 visible bands 6 | Results in 3 visible bands 7 |
| (6) Largest band ~600 bp  P. concolor | Largest band <500 bp  P. perproxima |
| (7) Cut with restriction enzyme NsiI: | Results in 2 bands Uncut |

Table 3
Molecular key to nine *Psytallia* species based on restriction digestion of a 709 bp (including PCR primers) section of the COI region of mtDNA with four restriction endonucleases.
3.3. Phylogenetic analyses

The parsimony and maximum likelihood analyses produced similar results to the Neighbor-Joining tree, with the same 10 groups identified (Figs. 2 and 3). Populations originating from central Kenya (K–N), Namibia (O), and South Africa (P) (P. humilis in Table 1) clustered together to form a sister-group relationship with P. concolor from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E). Similarly, there was strong support for a sister-group relationship between the populations from the Mediterranean region (A–E).

4. Discussion

Ten distinct groups were recognized using sequence data from the 28sD2 and COI regions. In general, the levels of divergence among distant populations of most of the groups we examined were surprisingly small. Populations identified as P. concolor from the Mediterranean region (A–D), including those from the Canary Islands (E), clustered nicely together in our phylogenetic analyses (Fig. 3) and were easily separated, on the basis of COI sequences, from all other populations of Psyttalia examined in this study. Mediterranean populations of P. concolor were most closely related to those of P. humilis originating from coffee infested with Ceratitis capitata in central Kenya (K–N), and from wild olives infested with B. oleae (and more rarely another species of Bactrocera) in Namibia (O) and South Africa (P). Populations of P. lounsburyi (Q&R), a species which has distinctly darker coloration, fell into a second clade along with otherwise pale-colored populations of P. cosyrae (G–I), P. perproxima (S–U) and P. cf. perproxima (J) (Fig. 3). Our data suggest a more rapid divergence between East and West African populations of P. cosyrae than equivalent populations of P. perproxima, which is somewhat unexpected, but may simply reflect different frequencies of introductions via imported fruits, though there is no empirical evidence for this. A third clade is formed by Kenyan populations of P. phaeostigma (V) and those of P. n. sp. (F) (Fig. 3). Finally, the population of P. ponerophaga from Pakistan formed the most genetically distinct group in the P. concolor species complex (Table 2; Fig. 3).

4.1. Application of available names

Problems associated with application of species names in the Psyttalia concolor complex have been discussed at some length (Fischer, 1958, 1972; Wharton and Gilstrap, 1983; Kimani-Njogu...
et al., 2001) and are reviewed below with respect to the populations we examined. The application of names to groups or populations is unfortunately a slightly different question than the determination of their status as valid (e.g. reproductively isolated) species. We have good evidence (Kimani-Njogu et al., 2001; Billah et al., 2007) that individuals from at least some of our populations are reproductively compatible with individuals from other populations, despite the genetic differentiation shown here. In small arenas, males will successfully inseminate females, resulting in viable female offspring. However, behavioral traits, or perhaps a more cryptic mechanism such as conspecific sperm precedence (Howard, 1999), may be acting to maintain reproductive isolation in the field. Given the genetic differentiation shown in this study, this seems likely, particularly in the case of sympatric populations of Psyllia from coastal Kenya. Nothing is known in this regards, however, and a study of the isolating mechanisms in these multivoltine species would go a long way towards answering the question of whether these represent several distinct species or populations of a few widespread species.

For several of the populations in our study, the application of names is straight-forward. Psyllia concolor is the oldest available name for the species examined in this study, and is readily applicable to populations from the Mediterranean region (A–E) since P. concolor was originally described from Tunisia. Psyllia lounsburyi is distinct morphologically, largely because of its darker coloration, and its identity has never been questioned. Populations of P. lounsburyi from Kenya (Q) and South Africa (R) have identical 28sD2 sequences and only a 2 bp difference in the more rapidly evolving COI gene. Additional studies on the differentiation among populations of P. lounsburyi are being pursued by M.C. Bon at the USDA lab in Montpellier, France. Psyllia ponerophaga is known only from Pakistan, where it was originally described by Silvestri (1916). Our material (W) comes from the type locality in Pakistan, reared from the same host and host plant noted by Silvestri (1916), and the name P. ponerophaga can, therefore, be applied without hesitation to our samples. This species has the most divergent COI sequences relative to other populations (Table 2), and although Silvestri noted a distinctive wing vein feature that distinguishes

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Fig. 3. Maximum-likelihood tree for a partitioned nucleotide model for concatenated 28sD2 and COI sequences. Non-parametric bootstrap support based on 1000 iterations and presented as percentages.
P. ponerophaga from P. concolor, this character unfortunately proved to be variable in the material at hand. Finally, the population of Psyttalia from coastal Kenya reared from fruits of Lettoviatus (F) is also distinctive genetically (though not morphologically). There is currently no available name that can be applied unequivocally to this population, and we consider it a separate, but as yet undescribed species on the basis of the molecular results as well as the biological differences noted below.

Of the available names in the genus Psyttalia, at least four are definitely applicable to the remaining populations under consideration here. These are P. humilis, P. cosyrae, P. perproxima, and P. phaeostigma. Other available names may eventually need to be considered because of issues of priority, but are presently excluded from consideration here due to host associations, geographic considerations, or minor morphological differences. The most important of these, from an olive fly control perspective, is Psyttalia dacicida (Silvestri, 1912), described from olive fly in Eritrea. The name has been applied to specimens reared from olives in South Africa (Neuenschwander, 1982), but unfortunately we have no material from Eritrea for molecular comparisons.

Silvestri (1913) described Psyttalia humilis from Medfly-infested fruit near Cape Town, South Africa, and this species was one of several successfully introduced to Hawaii to suppress Medfly populations (Back and Pemberton, 1918; Clausen, 1978). Since our material from South Africa (P) and Namibia (O), reared from olive fly, is virtually indistinguishable morphologically and genetically from material from Kenya (K–N), reared from Medfly, P. humilis is an available name appropriate for the entire group (Table 1). As previously noted, under caged conditions, individuals from these sub-Saharan populations are fully reproductively compatible with P. concolor from the Mediterranean region (Kimani-Njogu et al., 2001; Billah et al., 2007). Thus, the available evidence is inadequate for unambiguously deciding whether P. humilis is sufficiently distinct from P. concolor to call it a separate species. However, as there is a practical issue of distinguishing sub-Saharan populations from those originating from the Mediterranean region, in both past and on-going biological control programs, we suggest the name P. humilis can be used for the sub-Saharan populations studied here, and those which have been referred to as P. cf concolor in some of the previous publications using material from these same populations (e.g. Wharton et al., 2000; Kimani-Njogu et al., 2001).

Wilkinson (1927) described Psyttalia cosyrae from specimens reared from Ceratitis cosyra in Tanzania. Our samples, initially reared from C. cosyra in Kenya before being cultured in the laboratory (G), match Wilkinson’s description and type specimens and have, therefore, been referred to as P. cosyrae in previous studies (Mohamed et al., 2003, 2007). Our data show that in Kenya, wasps with long ovipositors that attack mango-infesting C. cosyra (H) are genetically distinct from those that attack cucurbit-infesting D. ciliatus (V), and that these in turn are genetically distinct from smaller wasps with consequently shorter ovipositors that were collected from wild fruits in coastal Kenya (S&IT) and from coffee in West Africa (U). Given this information, we tentatively assign the name P. cosyrae to material reared from the African mango pest, C. cosyra, the name P. phaeostigma to cucurbit pests, and the name P. perproxima to a mixture of populations from East and West Africa. Ovipositor length, while useful to some extent in differentiating species of Psyttalia, and in initial sorting of field-collected material, has to be used with caution because of difference in apparent size when reared on different hosts (Billah et al., 2005).

As with the populations of Psyttalia from coastal Kenya reared from tephritids infesting Lettoviatus (F), we have no applicable name for populations from coastal Kenya attacking tephritids infesting fruits of Flagellaria (J). The Psyttalia from Flagellaria is genetically distinct from the other groups treated here, and falls in the middle of the clade containing P. lounsburyi, P. cosyrae, and P. perproxima (Figs. 2 and 3). As its placement in our analysis is equivocal, we refer to it here as P. cf. perproxima, thus associating it with the oldest available name for predominantly pale-colored species in this clade.

4.2. Diagnostics

Restriction digests of a 709 bp region (includes PCR primers) of the COI region enabled complete separation of the nine species identified in this study, and in addition, allowed the further separation of P. cosyrae populations from Kenya and Benin (Table 3). However, P. cosyrae is the exception rather than the rule, and the mitochondrial DNA key presented here is unable to separate individual populations of the other eight species. This reveals a limitation of the key; it is context specific. We have designed the key to work well at the species-level. Thus, in order to separate individual populations within a species the key must be supplemented by sequencing of the COI region (see GenBank accessions). In turn, this reveals another limitation of the diagnostic methods presented here. Our mtDNA key may only be applicable to individuals collected from the respective geographical areas of each of the groups included in our study. To apply the key to Psyttalia populations from outside of the sampled range of the present study assumes that the variation shown here is indicative of all populations of each species/group. This seems unlikely to be the case given the levels of intra-specific polymorphism in the COI sequences displayed in the present study (e.g. P. humilis, Table 2). Thus, sequencing of both the COI and 28S D2 regions is recommended for populations of Psyttalia that fall outside the geographical boundaries of the current study. Furthermore, to enhance the deposition of any type material of such populations in museum collections, we strongly encourage future workers to also deposit complimentary sequences in GenBank.

4.3. Implications for biological control

The existence of several genetically distinct and recognizable populations should facilitate exploration of the potential for using members of the P. concolor species complex for reducing populations of Medfly and olive fly in different habitats. For example, our results suggest that P. concolor, P. humilis, and P. perproxima have populations that are locally adapted to certain hosts, host plants, and climatic regimes. Medfly is notorious for its broad host range (Copeland et al., 2002) and ability to occupy a wide variety of habitat types, especially when competing tephritids are scarce or absent. Though a specialist feeding only on olives, olive fly has rapidly dispersed throughout California following its introduction, and now occupies several different climatic zones (Collier and Van Steenwyk, 2003). The results presented here, while useful for Medfly biocontrol, are more immediately applicable to on-going work on the biological control efforts against olive fly in California (Sime et al., 2006a,b,c, 2007; Daane et al., 2008).

Our results may provide a mechanism for evaluation of efficacy of different source populations. The usefulness of these markers depends to a large extent on the reproductive compatibility between the different released populations. If two populations, differing in their mitochondrial markers, are completely reproductively compatible, the mitochondrial markers can only be used to determine the rate at which the released generation can find hosts. For instance if parasitoids from a variety of different upland and coastal sources are released simultaneously against a polyphagous target pest occupying a mosaic of habitats, we can determine the effectiveness of the different populations by determining their rates of parasitization of “trap hosts” placed in these habitats. The identity of parasitoids that emerge from the “trap
hosts” can be determined using our mtDNA key or sequencing the COI region. However, these markers would only be useful for a limited number of generations, because if the released populations do interbreed in the field, the mitochondrial marker loses its link with the nuclear genome, and will quickly come to reflect only the relative release rates of the different source populations. Under laboratory conditions, Kimani-Njogu et al. (2001) and Billah et al. (2007) were able to obtain viable female offspring from crosses of Psyttalia populations from Kenya and Italy, including successful hybridization of species with long and short ovipositors. However, the experimental crosses were done in small vials, in the absence of conspecific members of the other sex, and hence, did not consider the possibility of pre-copulatory mate choice (Andersson, 1994) or conspecific sperm precedence (Howard, 1999) as an effective barrier to hybridization. Thus, it is unknown whether such crosses would occur under field conditions. The level of genetic differentiation found here for populations from different host plant families in coastal Kenya suggest that there is some mechanism for mating isolation in the field.

The situation is of course different if there is a reproductive barrier of some sort between the different populations. Under these circumstances, mtDNA makers may be very useful to determine the origin of the established population. Our key could then be useful for determining whether any individuals of Psyttalia recovered from olive fly in California originated from either Kenya or Italy, the two sources for previous releases in that state (Yokohama et al., 2005; Sime et al., 2006c). Alternatively, parasitoids from native populations in subsaharan Africa may be released in areas where P. concolor from Mediterranean sources has already been released. This would be directly applicable to releases of “new” Psyttalia populations against olive fly in California, and may also be relevant to possible releases against Medfly in Guatemala and Hawaii.

Finally, the discovery of a genetically distinct population of Psyttalia reared from C. rosa in fruits of Lettowianthus in Kenya is somewhat surprising. Ceratitis rosa, known as the Natal fly in subsaharan Africa and nearby Indian Ocean islands, is a polyphagous species (Copeland et al., 2006) that in some areas is a more serious pest of edible fruits than Medfly (Annecke and Moran, 1982; Duyck et al., 2004). In Kenya, the Natal fly normally encapsulates Psyttalia (Mohamed et al., 2003, 2007), and the discovery of a population that is apparently able to avoid encapsulation is a significant find.

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