



Semiochemical Exploitation of Host-Associated Cues by Seven *Melittobia* Parasitoid Species: Behavioral and Phylogenetic Implications

Jorge M. González¹, Dakota Camino¹, Sabrina Simon² and Antonino Cusumano^{3*}

¹ Department of Plant Sciences, California State University, Fresno, Fresno, CA, United States, ² Biosystematics Group, Wageningen University and Research, Wageningen, Netherlands, ³ Laboratory of Entomology, Wageningen University and Research, Wageningen, Netherlands

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*Correspondence:

Antonino Cusumano
cusumanoantonino@tiscali.it

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Chemical compounds (infochemicals or semiochemicals) play an important role both in intra-specific and inter-specific communication. For example, chemical cues appear to play a key role in the host selection process adopted by insect parasitoids. In recent years significant advances have been made in order to understand the chemical ecology of insect parasitoids. However, little information is available about the evolution of semiochemical use in the host location process of insect parasitoids. Here we investigated the strategy adopted by seven closely related parasitoid species in the genus *Melittobia* when foraging for four different suitable hosts. By using an integrated approach that combined olfactometer bioassays and phylogenetic investigations, we found that: (1) exploitation of host-derived semiochemicals is widespread in the *Melittobia* genus; (2) there is specificity of attraction toward the different host species tested; in particular, the early-branching species in the *Melittobia* genus are attracted to odors associated with leaf cutting bees (*Megachile rotundata*) whereas the most-diverged species are attracted to odors associated with solitary mud dauber wasps (*Trypoxylon politum*). Regardless of the phylogenetic relationships, no *Melittobia* species exhibited attraction toward odors of factitious laboratory hosts (i.e., the flesh fly *Sarcophaga bullata*). Interestingly, five *Melittobia* species are also attracted by odors associated with honeybees hosts which indicate that these parasitoids could be potential pests of honeybees. Our study shed light on the host location within the *Melittobia* genus and represents a first attempt to understand semiochemical use in an evolutionary perspective in the context of parasitoids' foraging behavior.

Keywords: infochemicals, parasitic wasps, host location process, Hymenoptera, Eulophidae

INTRODUCTION

Semiochemicals are important sources of information that mediate ecological interactions in organisms at different degree of evolution across microorganisms, plants, arthropods, and vertebrates (Hildebrand, 1995; Carde and Millar, 2004). Semiochemicals play a role both in intra-specific and inter-specific communication (Dicke and Grostal, 2001; Wyatt, 2003). Within a species,

semiochemicals can be exploited to find and choose a mate, to mark the territory, for kinship recognition or to inform others of danger. Between species, semiochemicals can be used by natural enemies to locate preys/hosts and are widely exploited by parasitoids, i.e., insects whose eggs and larvae develop inside the body of other arthropods whereas the adult is free-living (Godfray, 1994; Fatouros et al., 2008; Colazza et al., 2014). In fact, for parasitoids, there is a direct link between host encounter and the production of offspring. Consequently, natural selection is expected to act strongly on parasitoids' host finding efficiency (Vet, 2001). Species that display high host finding efficiency may have an advantage when competing with antagonistic parasitoid species that are searching in the same habitat, especially if hosts are scarce (Harvey et al., 2013; Cusumano et al., 2016).

In recent years significant advances have been achieved toward understanding which cues are used by female parasitoids to locate hosts (Fatouros et al., 2005; Hilker and Meiners, 2006). Parasitoids can exploit host-associated cues including frass, honeydew, wing scales, footprints, silk and mandibular gland secretions (Chabi-Olaye et al., 2001; Thibout, 2005; Mehrnejad and Copland, 2006; González et al., 2011; Iacovone et al., 2016). In addition, parasitoids attacking herbivores rely widely on herbivore-induced plant volatiles (Mumm and Dicke, 2010). However, less is known on the evolutionary history of semiochemicals use in the context of host location by insect parasitoids. Theoretical models on the evolution of host selection have been developed but these models focused on the spatial distribution on host resources and do not take into account the exploitation of semiochemicals when parasitoids search for hosts (Charnov and Stephens, 1988). Phylogenetic approaches have been useful to investigate several evolutionary questions including historical pattern of host shifts (Cook et al., 2002) and host range specialization (Stireman, 2005) but the evolution of semiochemical use has never been considered in these studies. To implement data on semiochemical exploitation into phylogenetic analyses, the host location process of multiple closely related species must be studied. This was the purpose of this study, in which we used an integrated approach to investigate the host finding process of different parasitoid species within the genus *Melittobia* (Hymenoptera: Eulophidae), inferred their phylogenetic relationships and traced host location process on the phylogeny.

Melittobia wasps are small (1.0–1.5 mm) ectoparasitoid idiobionts that develop gregariously on their hosts (Matthews et al., 2009). They exhibit sexual dimorphism and females are polymorphic (short-winged and long-winged; González et al., 2004a; González and Matthews, 2005a). Long-winged females, also called macropterous females, are the dispersal individuals in all *Melittobia* species, while some non-dispersing short-winged females, also known as brachypterous females, may be produced in order to fully exploit large high quality hosts (Matthews et al., 2009). van den Assem et al. (1982) and Dahms (1984) placed all *Melittobia* species in four species-groups based on male displays during courtship and morphological characteristics, respectively. According to Tanner et al. (2011), the 12 known *Melittobia* species are classified in four phylogenetically-distinct groups ranging from most primitive to more advanced in the

following order: *assemi* species-group (2 species), *hawaiiensis* species-group (2 species), *clavicornis* species-group (1 species), and *acasta* species-group (7 species). *Melittobia* wasps tend to be polyphagous and they are usually recorded as natural enemies of solitary wasps and bees, such as mud dauber wasps in the genera *Trypoxylon* (Crabronidae) and *Sceliphron* (Sphecidae) and several cavity-nesting bees including important pollinators such as megachilid leaf-cutter bees (Krombein, 1967; Matthews et al., 2009). *Melittobia* have been also occasionally recorded to attack honey bees as well as several insects from different orders and can reproduce fine in factitious hosts (i.e., flesh flies) (Matthews et al., 2009). The ancestral host of *Melittobia* species is not known but it is possible that some solitary cavity-nesting bees which nested in aggregated situations are closely related to the original natural hosts, from which the host range could have been expanded to include solitary wasps such as mud dauber wasps which are notable because females tend to build nests in clusters (Malyshev, 1968; Cross et al., 1975; Molumby, 1995). Up-to date, only the host location process of 2 species of the *acasta* species-group (*M. digitata* and *M. acasta*) has been investigated showing that long-winged females are attracted by volatile semiochemicals emitted by the cocoons of mud dauber wasps (Cusumano et al., 2010) and megachilid leaf-cutting bees (Silva-Torres et al., 2005a,b; Glasser and Farzan, 2016) which envelop the host prepupa, i.e., the host stage attacked. However, it is not known whether the other *Melittobia* species have evolved a similar host location strategy based on exploitation of host-associated chemical cues.

In this study we first investigated the host location process of seven different *Melittobia* species belonging to the four species-groups using a comparative approach based on olfactometer experiments and reconstructed evolutionary relationships in terms of host odor attraction among seven *Melittobia* species. We explored the behavioral responses of those wasps within four species-groups toward host-associated volatiles emitted by both naturally parasitized hosts (the organ-pipe mud dauber, the alfalfa leaf-cutter bee and the honeybee) and factitious hosts (flesh flies). We hypothesized that the different species of *Melittobia* exploit host-specific semiochemicals when foraging for natural hosts. On the contrary no attraction to host-associated odors is expected for factitious hosts. To the best of our knowledge, this study represents the first attempt to investigate semiochemical use within a taxonomic unit to shed light into the evolutionary history of parasitoids' host location process.

MATERIALS AND METHODS

Insect Cultures Parasitoids

All *Melittobia* species (*assemi* group: *M. assemi* and *M. sosui*; *hawaiiensis* group: *M. cf. hawaiiensis* and *M. australica*; *clavicornis* group: *M. clavicornis*; *acasta* group: *M. acasta* and *M. digitata*) were reared on naked prepupae of *Trypoxylon politum* from stock cultures maintained at the Department of Plant Science facilities, California State University, Fresno, in Fresno, California. Cultures were established from material originally maintained at the University of Georgia, Department

of Entomology, Athens, Georgia, and the Entomology Research Laboratory of Texas A&M University, College Station, Texas. Our stock cultures were maintained under controlled conditions in a growth chamber (25°C; 60 ± 10% R.H.; 14L:10D). Healthy hosts were collected from their nests; prepupae were excised from cocoons and exposed to parasitoids. Only long-winged females <5 days-old were used in the experiments. Because of their age and since they were taken directly from the cultures prior to their use in the bioassays, female parasitoids were assumed to be mated. About 1–2 h before bioassays, the *Melittobia* wasps were taken from cultures and individually placed in size 1 gelatin capsules (0.5 ml volume, 6.63 mm in diameter).

Hosts

The following hosts were cultured in order to be used in the bioassays:

Apis mellifera (Hymenoptera: Apidae)

Honeybees are rare hosts of *Melittobia* species but they have been reported to be attacked by *M. acasta* and other *Melittobia* species (Hobbs and Kronic, 1971; Erickson and Medenwald, 1979; Jelinski and Wojtowski, 1984; de Wael et al., 1995; González et al., 2004a; Matthews et al., 2009) (see Supplementary Table 1 for host-parasitoid associations reported in the field and/or in the lab). Combs containing open and recently capped cells with larvae and prepupae of honeybees were obtained from two sources: the No Walls Busy Bee Honey Farm, Fresno, CA, and T & A Farms, L.A., CA, USA.

Megachile rotundata (Hymenoptera: Megachilidae)

Alfalfa leaf-cutter bees can be attacked by *M. acasta* and other *Melittobia* species in the field, especially in areas where the bee is used for pollination (Hobbs and Kronic, 1971; Farkas and Szalay, 1985; González and Matthews, 2002; González et al., 2004a; Matthews et al., 2009). The bees were obtained as diapausing prepupae inside the cocoons from Pioneer Hybrid International, Namper, ID, USA.

Trypoxylon politum (Hymenoptera: Crabronidae)

Organ-pipe mud dauber wasps are natural hosts of *Melittobia* species and they are frequently found parasitized by *M. australica*, *M. acasta*, and *M. digitata* (González et al., 2004a,b; González and Matthews, 2005a; Matthews et al., 2009). Mud dauber wasps prepupae were obtained from nests collected at one of our traditional collecting sites (González et al., 1985; Matthews et al., 1985) (Watson Mill Bridge, near Comer and Carlton, Georgia, 34.0261° N, 83.0731° W). The wasps' prepupae remain in diapause and viable if kept in a refrigerator (8–12°C).

Sarcophaga (Neobellieria) bullata (Diptera: Sarcophagidae)

Flesh flies are factitious hosts of several species of *Melittobia* wasps and they are currently used in commercial production of *M. digitata* (Matthews et al., 2009). The flesh flies used in our trials were obtained from a commercial source (Carolina Biological Supply Company, Burlington, NC, USA).

All hosts were kept in a refrigerator at <10°C to maintain them, especially prepupae of mud dauber wasps, in diapause to avoid their development.

Stimuli Tested

Volatile semiocemicals emitted by different hosts of *Melittobia* were tested in dual choice conditions using always clean air as control. The treatments were: (1) *Apis mellifera* prepupae, which were removed from their cells with soft forceps; (2) *Megachile rotundata* prepupae inside cocoons which were cut at the tip with a micro scissor, to confirm the stage and quality of the hosts; (3) *Trypoxylon politum* prepupae enveloped inside cocoons gently broken at the subtruncate cap in order to open a small orifice to verify that the prepupae was healthy and not parasitized; (4) *Sarcophaga (Neobellieria) bullata* pupae inside their puparia.

Y-Tube Olfactometer Behavioral Assays

The response of macropterous females of seven *Melittobia* species toward host-related volatile semiocemicals was investigated using a Y-tube olfactometer made up of polycarbonate (stem: 90 mm long; arms: 80 mm long, with an angle of 130° in between; inner diameter: 15 mm) sandwiched between two glass sheets. A 10-mm diameter hole was drilled through the device into the end of each arm to allow the connection with air tubes and the introduction of the test wasp.

Medical-grade compressed air was passed through the tubes connected to both arms of the Y-tube. The incoming air was regulated by flowmeters at 35 ml per min, based on previously published information on similar tests done with *M. digitata* showing that the wasp's walking behavior was optimal at such a low rate (Cusumano et al., 2010). Before reaching the arms of the Y-tube apparatus, air was purified by a charcoal filter and humidified by bubbling into distilled water flasks. The Y-tube was illuminated from above with red fluorescent lights (40-W fluorescent bulbs covered with red tube guards) keeping visibility in accordance to parameters previously established by González et al. (1985, 2011) and Matthews et al. (1985). To avoid visual or physical cues, the host species tested were enclosed in a small nalgene cylinder (12 × 9 mm) with copper wire mesh (18 Standard US mesh) on both sides. The cylinder with the host test choice was randomly placed close to the edge of one of the arms, while an empty one was used as control in all bioassays and placed at the other arm. A female wasp was gently introduced inside the Y-tube olfactometer at the bottom of the stem and it was allowed to explore for 900s before being discarded.

The wasps' behavior was measured in terms of first choice, i.e., the first time wasps crossed a virtual line, defined in each arm at 4 cm distal to the bifurcation of the Y-tube olfactometer, and remained for at least 30 s. The bioassays were performed in blocks according to the stimuli tested (*T. politum*, *M. rotundata*, *A. mellifera*, and *S. bullata*). Test combinations with 7 different *Melittobia* species were randomized within each block. For each tested combination 46–54 successful replicates were performed, indicating that wasps were replaced if a no-choice was scored after 900 s. Between replicates the odor source was renewed and the position of test and control were switched to avoid possible biases between both arms. The walking behavior of every tested *Melittobia* wasp was observed with a video Camera (Logitech Carl Zeiss Tessar HD 1080P) connected to a PC (Dell Optiplex 790) with a Monitor (19", Model 1908FP, Dell), in order to observe the wasps away from the Y-tube and avoid possible interference from the observer on the walking behavior of the

Melittobia wasp being tested. The room was kept at 20°C and 60% RH. All experiments were conducted from 8:00 to 12:00 h and from 13:00 to 17:00 h.

In Y-tube olfactometer experiments, the number of wasp females that made a response was analyzed by χ^2 test. We tested whether the observed distribution of responding wasps significantly diverged from a 50:50 distribution which is expected if the wasps do not display any attraction toward the tested odors. All data were analyzed using STATISTICA7 software (StatSoft, 2001).

Evolutionary Relationships in Parasitoids' Host Location Mediated by Semochemicals Sampling

To reconstruct the evolutionary relationships of host location among the seven *Melittobia* species, available sequences of several *Melittobia* species as well as sequences from outgroup species were downloaded from NCBI GenBank (accession numbers JF924912–JF925004). We further extracted DNA from ethanol (98%) preserved *M. cf. hawaiiensis* species using the DNeasy Blood and Tissue kit from Qiagen. We amplified the Internal Transcribed Spacer (ITS) regions 1 and 2 of the nuclear rRNA using the primer sequences as provided in Tanner et al. (2011). The amplification profile was as follows: 5 min at 95°C, 35 cycles of 30 s at 95°C, 45 s at 58°C and 2 min at 72°C for elongation, and a final extension of 5 min at 72°C. We further amplified the Cytochrome Oxidase 1 (CO1) barcode region of the mtDNA using the universal invertebrate Folmer primers LCO1490–CO2198 (Folmer et al., 1994). The amplification profile was as follows: 5 min at 95°C, 35 cycles of 30 s at 95°C, 45 s at 50°C and 2 min at 72°C for elongation, and a final extension of 5 min at 72°C. PCR products were checked and the size was determined by agarose gel electrophoresis. PCR products were purified with Qiagen's QIAquick Gel Extraction Kit and sequencing reactions were carried out by Eurofins. The newly generated *M. cf. hawaiiensis* gene sequences are publicly available (GenBank accession numbers MG263513–MG263515).

Phylogenetic Analysis

Compared to the study of Tanner et al. (2011) we included the three newly generated sequences of *M. cf. hawaiiensis* in our taxon sampling for the phylogenetic analyses. In addition, we applied a more extensive maximum likelihood (ML) analyses including FreeRate models and performing independent tree searches (see below). First, individual gene alignments were generated using MAFFT v7.122b (Kato and Standley, 2013) and subsequently concatenated using FASconCAT v1.0 (Kück and Meusemann, 2010). The concatenated alignment was subjected to maximum likelihood (ML) and Bayesian analyses (BI). For the ML analysis we first used IQ-TREE v.1.5.0 (Nguyen et al., 2015) to perform an extended model selection that additionally includes FreeRate models (-m TESTNEWONLY) for each gene partition (-spp) (697 bp: CO1, 1,798 bp: ITS1, 726 bp: ITS2). The assumed model of nucleotide substitution was K3Pu+R3 for CO1, K2P+G4 for ITS1, and TIM3e+R3:

ITS2. We further selected the tree with the best log-likelihood score found in 50 completely independent tree searches using IQ-TREE v.1.5.0 (Nguyen et al., 2015; Chernomor et al., 2016). Support values for ML trees were estimated with 100 bootstrap replicates.

The BI analysis was conducted with MrBayes v.3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) similar to the approach as in Tanner et al. (2011) with ITS1 and ITS2 partitioned according to the general time-reversible model with gamma-distributed rate variation across sites (GTR + Γ), and CO1 partitioned according to the general time-reversible model with invariant sites and gamma-distributed rate variation across sites (GTR + I + Γ), and with all parameters independent across loci. The matrix was analyzed over 3,000,000 generations using four chains (one cold, three heated) and a sampling frequency of 100. The average standard deviation of the split frequencies was below 0.01 and the first 250,000 trees were discarded as the "burn-in." The remaining trees were assembled into a topology.

Ancestral Character Reconstruction and Evolutionary Relationships in Host Location

We used SIMMAP v.1.5 (Bollback, 2006) and Mesquite v.3.2 (Maddison and Maddison, 2017) to reconstruct the evolution of host preferences among the seven *Melittobia* species. In SIMMAP our model was constructed such that the bias parameter for the discrete characters was equal (1/k) prior, all states were unordered, and the gamma distribution prior had a value of 1.25 and a b value of 0.25 with 60 categories. In Mesquite we used Maximum likelihood algorithms to reconstruct character evolution with the default probability models. In these analyses, we used the wasps' behavior measured in terms of first choice, which was defined as described above.

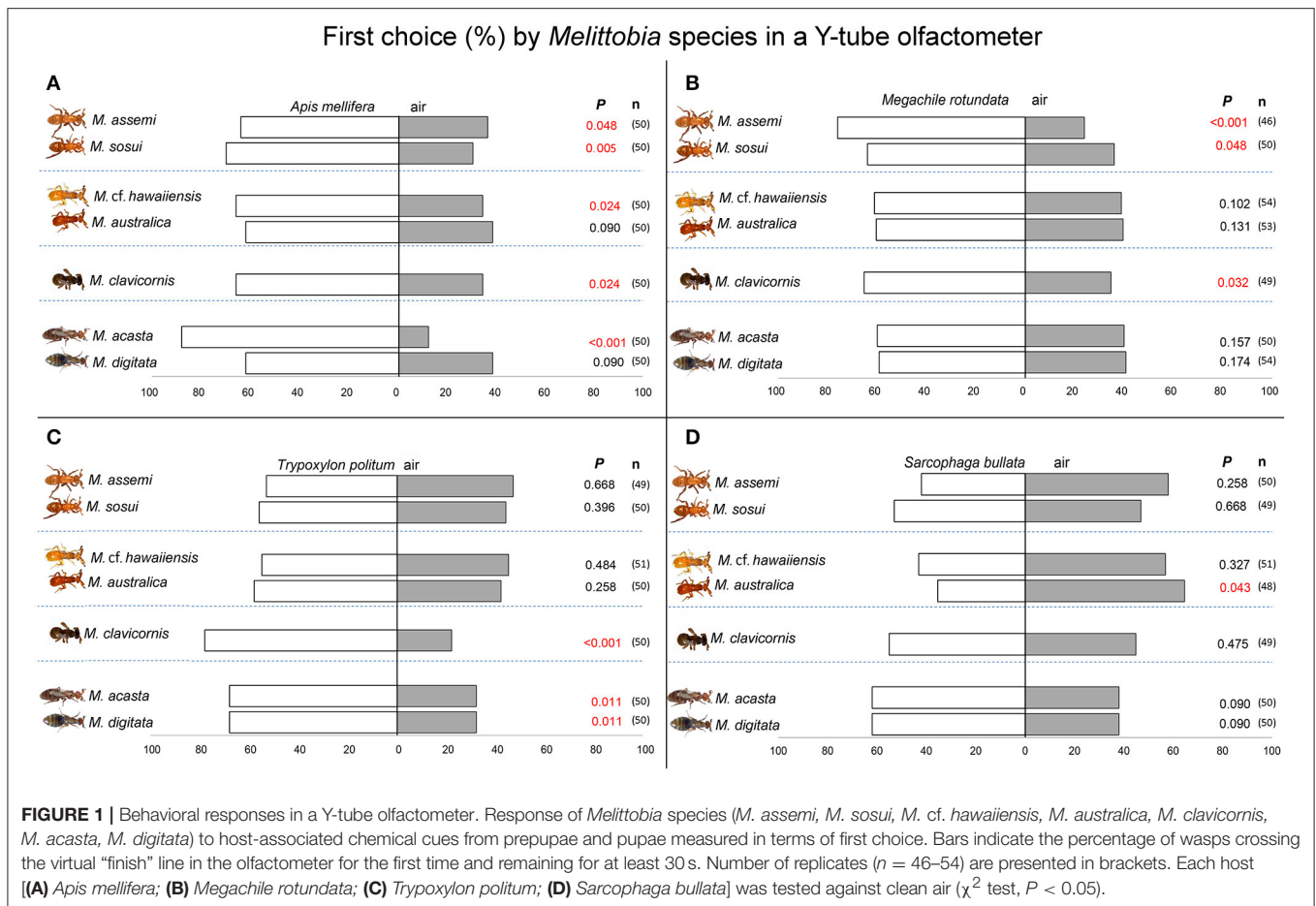
RESULTS

Olfactometer Experiments: Wasp Responses to Host Semochemicals *Apis mellifera*

Five species of *Melittobia* showed a significant attraction to volatiles released by prepupae of *A. mellifera* (Figure 1A). The early-branching *Melittobia* species (*M. assemi*: $\chi^2 = 3.92$, $df = 1$, $P = 0.048$ *M. sosui*; $\chi^2 = 8.00$, $df = 1$, $P = 0.005$) were attracted by host-associated volatiles. Among the more-diverged species, *M. cf. hawaiiensis* ($\chi^2 = 5.12$, $df = 1$, $P = 0.024$) and *M. clavicornis* ($\chi^2 = 5.12$, $df = 1$, $P = 0.024$) significantly preferred volatiles emitted by *A. mellifera* when tested against clean air. In the case of *M. acasta*, a species belonging to the most evolved group, such preference was highly significant ($\chi^2 = 28.88$, $df = 1$, $P < 0.001$). No significant attraction was found for *M. australica* ($\chi^2 = 2.88$, $df = 1$, $P = 0.090$) or *M. digitata* ($\chi^2 = 2.88$, $df = 1$, $P = 0.090$).

Megachile rotundata

Three species of *Melittobia* were significantly attracted by volatiles released by cocoons containing *M. rotundata* prepupae (Figure 1B). Interestingly only the early-branching species



(*M. assemit*: $\chi^2 = 12.52$, $df = 1$, $P < 0.001$; *M. sosui*: $\chi^2 = 3.92$, $df = 1$, $P = 0.048$) and the more diverged *M. clavicornis* ($\chi^2 = 4.592$, $df = 1$, $P = 0.032$) showed such attraction, whereas species belonging to the most diverged group (*M. acasta*: $\chi^2 = 2.00$, $df = 1$, $P = 0.157$; *M. digitata*: $\chi^2 = 1.85$, $df = 1$, $P = 0.174$) did not prefer volatiles emitted by *M. rotundata* when tested against clean air. Additionally species of the *hawaiiensis* group (*M. cf. hawaiiensis*: $\chi^2 = 2.67$, $df = 1$, $P = 0.102$; *M. australica*: $\chi^2 = 2.28$, $df = 1$, $P = 0.131$) were also not attracted.

Trypoxylon politum

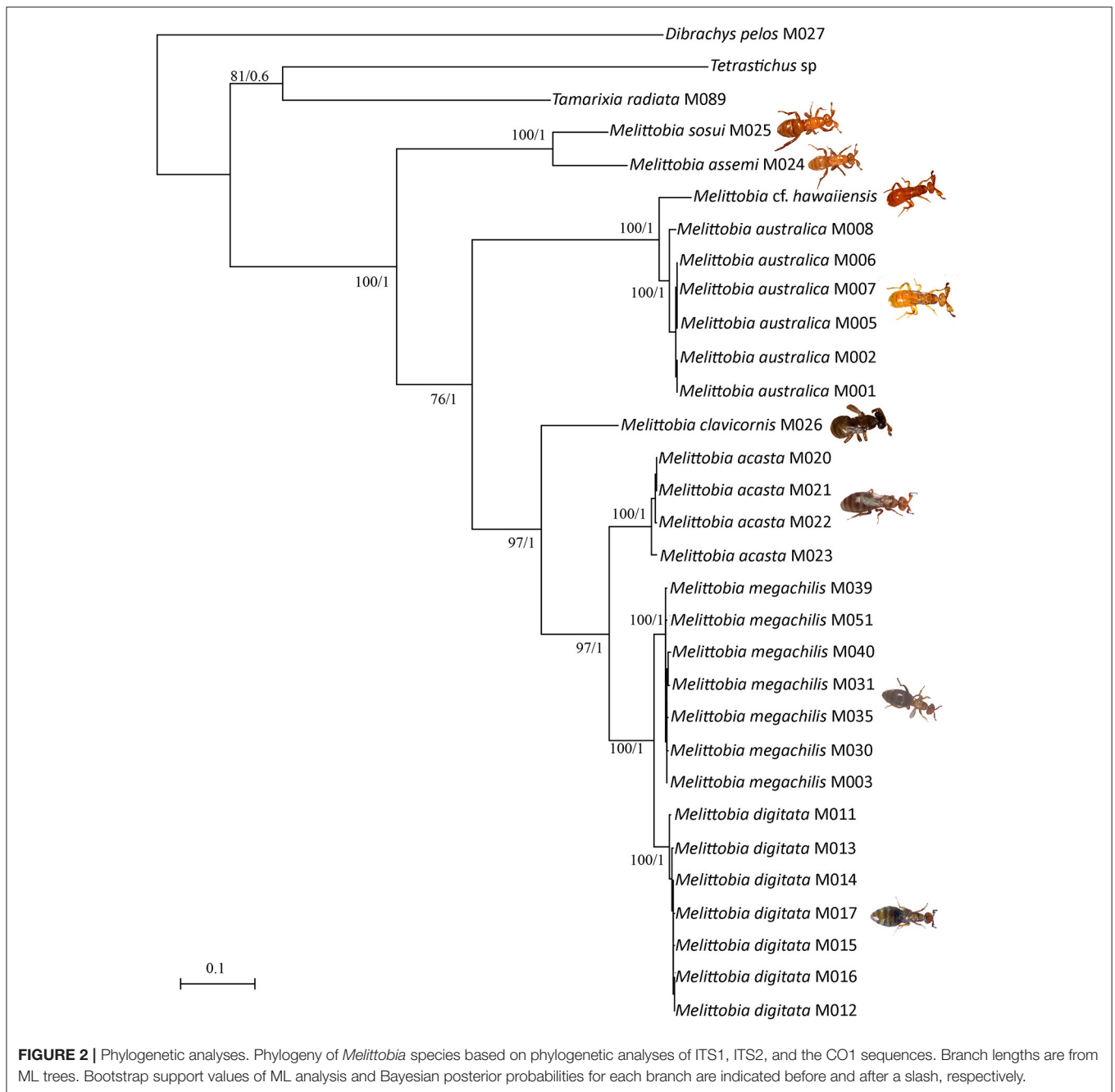
Three species of *Melittobia* significantly responded to volatiles emitted by prepupae of *T. politum* (Figure 1C). Interestingly, species at the bottom of the evolutionary scale such as the early branching *Melittobia* (*M. assemit*: $\chi^2 = 0.18$; $df = 1$, $P = 0.668$; *M. sosui*: $\chi^2 = 0.72$; $df = 1$, $P = 0.396$) and the species of the *hawaiiensis* group (*M. cf. hawaiiensis*: $\chi^2 = 0.49$; $df = 1$, $P = 0.484$; *M. australica*: $\chi^2 = 1.28$; $df = 1$, $P = 0.258$) did not show a behavioral response. On the contrary, the most diverged species (*M. acasta*: $\chi^2 = 6.48$; $df = 1$, $P = 0.011$; *M. digitata*: $\chi^2 = 6.48$; $df = 1$, $P = 0.011$) as well as *M. clavicornis* ($\chi^2 = 15.68$; $df = 1$, $P < 0.001$), significantly preferred volatile semiochemicals emitted by *T. politum* prepupae over clean air.

Sarcophaga bullata

No significant attraction toward volatiles emitted by *S. bullata* was shown by the seven tested *Melittobia* species (Figure 1D). In fact a slight, but significant, avoidance effect was found in *M. australica* ($\chi^2 = 4.03$; $df = 1$, $P = 0.043$) whereas the other species did not show any preference in the Y-tube olfactometer (*M. assemit*: $\chi^2 = 1.28$, $df = 1$, $P = 0.258$; *M. sosui*: $\chi^2 = 0.184$, $df = 1$, $P = 0.668$; *M. cf. hawaiiensis*: $\chi^2 = 0.961$, $df = 1$, $P = 0.327$; *M. clavicornis*: $\chi^2 = 0.51$, $df = 1$, $P = 0.475$; *M. acasta*: $\chi^2 = 2.88$, $df = 1$, $P = 0.090$; *M. digitata*: $\chi^2 = 2.88$, $df = 1$, $P = 0.090$).

Evolutionary Relationships in Parasitoids' Host Location Mediated by Semiochemicals

Our phylogenetic analyses further support the *Melittobia* species-group concept as proposed by van den Assem et al. (1982) and Dahms (1984) (Figure 2). Tanner et al. (2011) also supported based on molecular analyses the *M. assemit* species-group, confirming the sister-group relationship of *M. assemit* and *M. sosui*, the *M. acasta* species-group, confirming the sister-group relationship of *M. acasta* to *M. megachilis* and *M. digitata*, and the *M. clavicornis* species-group. Based on our more extensive maximum likelihood analyses, we could



robustly support these *Melittobia* species-group relationships. In contrast to Tanner et al. (2011), we were now also able to verify the *M. hawaiiensis* species-group, supporting a sister-group relationship of *M. australica* and *M. cf. hawaiiensis* due to the newly generated sequence information. On this regard, we are further investigating the latter population, which even though it is close genetically to *M. australica* (Figure 2) it diverges behaviorally from it (González and Parry, unpublished). Similar cases, treated as “intermediates” between *M. australica* and *M. hawaiiensis* have been previously reported by van den Assem et al. (1982) and Dahms (1984).

Our analyses further support the phylogenetic relationships among the *Melittobia* species-groups as shown by Tanner et al. (2011). The *M. assemi* species-group represents the early branching species-group within *Melittobia* and the *M. hawaiiensis* species-group is the sister-group to the clade comprising the *M. clavicornis* and the *M. acasta* species-group.

The results of the ancestral character reconstruction are shown in Tables 1, 2 and further in Figure 3. The reconstruction yielded no conflict between likelihood and Bayesian analyses (BA), but the likelihood approach (LA) reconstructed them with greater uncertainty (equivocal) than the Bayesian approach.

TABLE 1 | SIMMAP ancestral state reconstruction analysis.

Node	Control	<i>Trypoxylon politum</i>	Control	<i>Megachile rotundata</i>	Control	<i>Apis mellifera</i>
1	0.6139	0.3861	0.3920	0.6080	0.3066	0.6934
2	0.9243	0.0757	0.0845	0.9155	0.0830	0.9170
3	0.4150	0.5850	0.6111	0.3889	0.3719	0.6281
4	0.9761	0.0239	0.9835	0.0165	0.6160	0.3840
5	0.0755	0.9245	0.5943	0.4057	0.2263	0.7737
6	0.0226	0.9774	0.9274	0.0726	0.3460	0.6540

The probability of each host-associated semiochemical being exhibited by the ancestor represented at each node in the phylogeny.

TABLE 2 | Mesquite ancestral state reconstruction analysis.

Node	Control	<i>Trypoxylon politum</i>	Control	<i>Megachile rotundata</i>	Control	<i>Apis mellifera</i>
1	0.8688	0.1312	0.3689	0.6311	0.5000	0.5000
2	0.9823	0.0177	0.1004	0.8996	0.5000	0.5000
3	0.7826	0.2174	0.5869	0.4131	0.5000	0.5000
4	0.9722	0.0278	0.8906	0.1094	0.5000	0.5000
5	0.1044	0.8956	0.5055	0.4945	0.5000	0.5000
6	0.0146	0.9854	0.8741	0.1259	0.5000	0.5000

The probability of each host-associated semiochemical being exhibited by the ancestor represented at each node in the phylogeny.

The ancestral state reconstruction revealed both *A. mellifera*-associated odors and *M. rotundata*-associated odors as the potential ancestral host chemical cues exploited by the *Melittobia* species-group. However, while *M. rotundata*-associated odors are supported as the ancestral host chemical cues exploited by *Melittobia* for host location in both approaches (BA: 0.61, LA: 0.63), the support for *A. mellifera*-associated odors as the ancestral host chemical cues is based on the Bayesian approach (0.69) (see **Table 1**) while the likelihood approach is equivocal (0.5) (see **Table 2**).

DISCUSSION

Insect parasitoids can exploit a variety of stimuli while foraging for hosts among which chemical cues appear to play a major role (Godfray, 1994; Vinson, 1998; Fatouros et al., 2008; Colazza et al., 2014). In this study we show that macropterous females of *Melittobia* species clearly rely on semiochemical exploitation of host-associated cues when searching for hosts. Volatiles released by cocoons enveloping the host prepupae thus are not only relevant for *M. digitata* and *M. acasta* (Silva-Torres et al., 2005a; Cusumano et al., 2010; Glasser and Farzan, 2016), but these host semiochemicals appear to mediate the host finding behavior in several species within the genus *Melittobia*.

Interestingly, different *Melittobia* species displayed variation in terms of behavioral response toward odors emitted by leaf-cutter bees, mud dauber wasps, honeybees and flesh flies. The early-branching species (*assemi*-group: *M. assemi* and *M. sosui*)

were attracted to volatiles released from the alfalfa leaf-cutter bee *M. rotundata* and *A. mellifera*, suggesting that these bee odors could be related to those emitted by the ancestral original host attacked by *Melittobia* wasps (see **Table 2**). However it is most likely that the odors associated with alfalfa leaf-cutter bee *M. rotundata* are related to those associated with the ancestral host since *A. mellifera* is generally attacked by *Melittobia* spp. only when colonies do not possess a healthy status (Matthews et al., 2009). On the contrary the most-diverged species (*acasta*-group: *M. acasta* and *M. digitata*) responded to semiochemicals released by cocoons enveloping *T. politum* prepupae, a common host frequently parasitized by several *Melittobia* species in the field (Matthews et al., 2009). These results are unlikely affected by pre-imaginal experience as previously shown that the rearing history did not influence host choice (Silva-Torres et al., 2005b). Previous studies have also shown that learning does not affect the foraging behavior of macropterous females at least in *M. digitata* (González et al., 2011). Organ-pipe mud dauber hosts represent large, high quality hosts compared to leaf-cutter bees (González and Matthews, 2002) and a single *Trypoxylon* prepupae can support the production of short-winged females yielding more than 400 offspring (most of them long winged females) per host (González et al., 2013). Thus a high host location efficiency toward mud dauber wasps seems adaptive as it can maximize the reproductive success of *Melittobia* wasps, taking also into account that a macropterous female usually locates, parasitizes and/or superparasitizes only a single host during her lifetime (Freeman and Ittyeipe, 1993; Matthews et al., 2009). Across the more-diverged species, (*clavicornis* group: *M. clavicornis*) wasps are attracted by both odors from alfalfa leaf-cutter bee and from organ-pipe mud dauber wasp; however the host location strategy adopted by species in the *hawaiiensis* group (*M. australica* and *M. cf. hawaiiensis*) is still puzzling as they apparently do not exploit volatile semiochemicals emitted by the cocoons enveloping prepupae of *T. politum* or *M. rotundata*. It is possible that species in the *hawaiiensis* group exploit different cues to locate their hosts, for example cues produced by a stage different than that attacked (infochemical detour *sensu*, Vet and Dicke, 1992). The strategy based on cues indirectly associated with the host presence appears widespread among parasitoids, which often exploit host semiochemicals from the adult stage (Noldus et al., 1991; Colazza et al., 1999; Fatouros et al., 2005). In the case of solitary bees and wasps, the adult female constructs the nest for the offspring with the aid of her mouthparts; thus it has been suggested that volatiles from the nest under construction, which are contaminated with chemicals associated with female mouthparts, can also be important in localizing suitable hosts (Cusumano et al., 2010). Unfortunately, we could not test parasitoids' attraction toward odors emitted by nest under constructions due to the technical difficulties associated in retrieving such nests. Further studies should be conducted to investigate whether the host location strategy of species in the *hawaiiensis* group diverged from the other *Melittobia* species and whether it is indeed based on indirectly associated chemical cues. Testing the nest material will be beneficial to fully understand the host location strategy adopted by *Melittobia* macropterous females when foraging for hosts in nature.

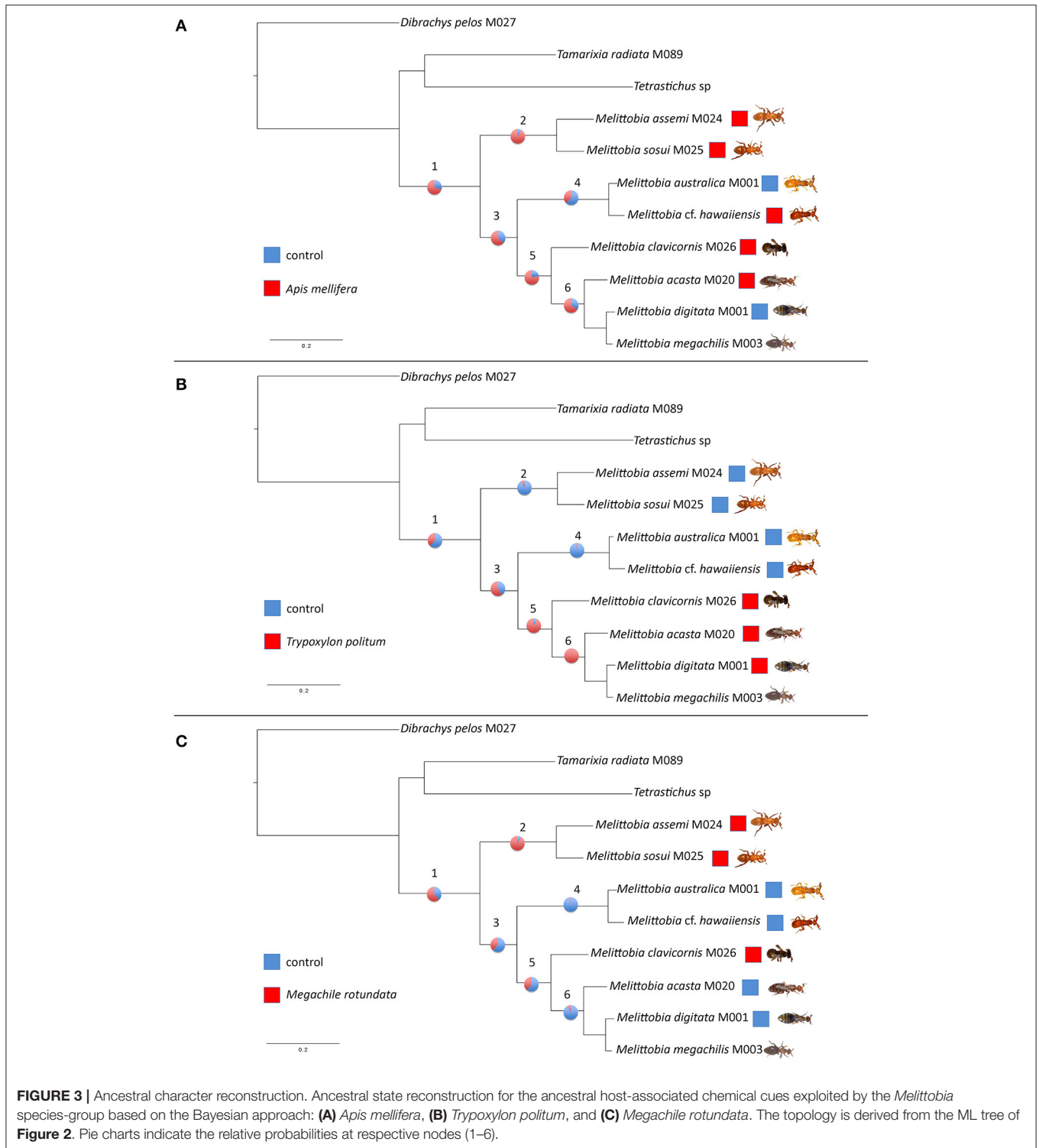


FIGURE 3 | Ancestral character reconstruction. Ancestral state reconstruction for the ancestral host-associated chemical cues exploited by the *Melittobia* species-group based on the Bayesian approach: **(A)** *Apis mellifera*, **(B)** *Trypoxylon politum*, and **(C)** *Megachile rotundata*. The topology is derived from the ML tree of **Figure 2**. Pie charts indicate the relative probabilities at respective nodes (1–6).

As expected, we found no attraction by any *Melittobia* species toward odors emitted by the factitious host *S. bullata*, indicating that macropterous females did not evolve the ability to exploit semiochemicals from the pupa of this dipteran species. However, in the laboratory, *Melittobia* species attack dipteran (i.e.,

Calliphoridae, Sarcophagidae, Tephritidae), lepidopterans (i.e., Tortricidae), and coleopteran (Tenebrionidae) species, but in the field some *Melittobia* species have been also found attacking fruit flies (Tephritidae) and even lepidopterans (i.e., Tortricidae) and roach eggs (i.e., *Periplaneta americana*) (González et al., 2004b,

2013; González and Matthews, 2005b; Matthews et al., 2009). This tremendous plasticity may serve in the field to parasitize satellite flies (Sarcophagidae) and bee flies (Bombyliidae) that sometimes infest the nest of their preferred hosts, *Trypoxylon* mud daubers (Matthews et al., 2009). In our experiments we were surprised to find that five species of *Melittobia* showed a significant preference for volatiles released by prepupae of *A. mellifera*, regardless of the wasps' phylogenetic relationships. This finding raises the concern of *Melittobia* species being potential pests of pollinators as the majority of the tested species can exploit host semiochemicals to locate honeybees. However, the risks for honeybee colonies appear to be low even though *Melittobia* parasitoid can reproduce fine on honeybees' pupae and prepupa (Cónsoli and Vinson, 2002a,b). In fact, whereas there have been reports of *Melittobia* parasitizing declining hives in Europe or queen producing hives in North America (Hobbs and Kronic, 1971; Erickson and Medenwald, 1979; Jelinski and Wojtowski, 1984; de Wael et al., 1995), it has been shown that *Melittobia* are unable to get past the honeybee hives' effective colony defense and hygienic behavior when colony possesses a healthy status (Whitfield and Cameron, 1993). However, the risk as pests of pollinators is higher for non-*Apis* pollinators such as leaf-cutter bees (Megachilidae) because of the *Melittobia*'s high reproductive potential, the ability to enter nests under construction and a strong female-biased sex ratio (typically 95% of the offspring consist of females) (Matthews et al., 2009).

In conclusion, we have shown that host-associated volatile cues play an important role in the host location of seven *Melittobia* species. We have also highlighted the specificity of semiochemical exploitation across the seven *Melittobia* species. We are aware that the performed phylogenetic analysis to reconstruct the evolution of host location within the genus *Melittobia* has some limitations: for instance, not all the hosts naturally attacked or not all the parasitoid species present within the genus have been tested in this study. However, despite these limitations, this study represent an interesting new

piece of information about the ecology of *Melittobia* species and can be the basis to extend knowledge on semiochemical use in an evolutionary perspective in the context of foraging behavior of insect parasitoids. Future investigations should combine olfactometer experiments with Gas Chromatography-Mass Spectrometry analyses focusing on polar and apolar extracts of prepupae. These investigations should be conducted in the attempt to characterize the blend of semiochemicals that mediate host-attraction behavior and unravel if different *Melittobia* species exploit the same volatile compounds to locate common shared hosts.

AUTHOR CONTRIBUTIONS

JMG, DC, and AC conceived and designed experiments; JMG and DC performed experiments; SS and AC analyzed data; all authors interpreted results, drafted and revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2017.00172/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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