Plant Phytochemistry Influences the Niche Breadth of Oligophagous Parasitoids (Hymenoptera: Braconidae)

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Abstract

The order Hymenoptera is one of, if not the most, species-rich order of animals, primarily driven by the many parasitoid wasps and their unique arthropod hosts. As hosts are necessary for survival, host location and selection have direct fitness implications. Semiochemicals, also known as infochemicals, from either the host herbivore or the plant are just one of many cues that parasitoid wasps use to locate a suitable host. The reliability-detectability hypothesis suggests that infochemicals from the host herbivore are the most reliable indicators of host presence. However, as localized signals, infochemicals are less detectable by parasitoids. Alternatively, infochemicals from plants are detectible over longer distances, although these signals are less reliable indicators of host herbivore presence. In this study, we explore the occurrence of infochemicals emitted by plants and their association with parasitoid host selection. We identified 112 phytochemical compounds extracted from Eucalyptus leaves that had been attacked by leaf beetle herbivores (Chrysomelidae) which had in turn been parasitized by Eadya larval-parasitoid wasps (Braconidae). Using several phylogenetic and statistical approaches, we conclude that host selection in two species of Eadya wasps is heavily influenced by infochemicals from the plant 1st trophic level. With this evidence, we propose amending the reliability-detectability hypothesis to include direct interaction between the 1st and 3rd trophic levels in a scenario where an oligophagous parasitoid utilizes oligophagous hosts.

Introduction

Hymenoptera (bees, wasps, and ants) are arguably the most species-rich group of animals on earth, in large part due to the sheer diversity of parasitoid wasps within the order as driven by the close associations these wasps have with their host arthropods (Forbes, Bagley, Beer, Hippee, & Widmayer, 2018). Parasitoid wasps lay their eggs either *on* (ectoparasitoids) or *in* their hosts (endoparasitoids), with some arresting the development of their hosts upon oviposition (idiobionts), whereas others allow their host to continue development during wasp larval growth (koinobionts) (Askew & Shaw, 1986). The level of host specialization varies among parasitoid wasps. Some species are monophagous, specializing on a single arthropod host species (oligophagous), while others utilize a wider, albeit often closely related, range of arthropod hosts, (host-flexible), Koinobiont endoparasitoids are likely to be more specialized because developing wasp larvae must survive through host development and must successfully defend against the host's immune response to parasitism(Beckage & Gelman, 2004; Vinson & Iwantsch, 1980).

As a host is required for parasitoid survival and reproduction, there are direct fitness consequences to selecting a host (Charnov & Skinner, 1984; Kouamé & Mackauer, 1991; Wang & Messing, 2004). Further, the process of selecting and locating a host differs depending on the level of host specialization of the parasitoid wasp (Bertoldi, Rondoni, Brodeur, & Conti, 2019; Vet & Dicke, 1992; Vinson, 1976). Although there are many

factors that may influence host selection in parasitoids (Price, 1971), for host-flexible parasitoids some host species may provide a fitness advantage relative to others. This begs the question: where a host preference exists, how do host-flexible parasitoids locate and select their preferred hosts?

Semiochemical cues (infochemicals) emitted from the host herbivore or plants, also referred to as the plant host complex (PHC), are one way parasitoid wasps may locate a viable host (Tumlinson, Lewis, & Vet, 1993; Vet & Dicke, 1992). Among infochemicals, there are three main classifications: (1) Kairomones that benefit the receiver, (2) allomones which benefit the sender, and (3) synomones that benefit both the sender and receiver (Brown Jr, Eisner, & Whittaker, 1970). For parasitoids, kairomones are often in the form of non-volatile oral secretions or feces from the host (Rutledge, 1996), although volatile pheromones may also be utilized. Synomones are typically plant-emitted volatiles and may be either herbivore-induced or constitutively produced (Hilker & McNeil, 2008; McCormick, Unsicker, & Gershenzon, 2012; Paré & Tumlinson, 1999; Tumlinson, Lewis, et al., 1993). The detection of these infochemicals by parasitoids can be innate or learned, although there are still relatively few studies documenting how host-flexible parasitoids use infochemicals for foraging and host acceptance (Steidle & Van Loon, 2003).

Vet and Dicke (1992) hypothesized that not all infochemicals are equal, and instead suffer from what they term the 'reliability-detectability problem'. Under this paradigm, host kairomones have high reliability because they are excellent indicators of host presence. However, they also have low detectability because they are typically localized and not detectable at a distance (Vet & Dicke, 1992). Due to their reliability, these infochemicals likely play an important role as a dependable indicator of the presence a specific host for monophagous parasitoids (Vet & Dicke, 1992). Vet and Dicke (1992) also contend that plant-emitted synomones travel much farther than host compounds making them more likely to be detected at a distance by foraging parasitoids, but are likely poor indicators of host presence and therefore unreliable. One hypothesized solution to the reliability-detectability problem is herbivore-induced synomones, infochemicals released from the plant in response to damage from herbivory termed the plant-host complex (PHC). Vet and Dicke (1992) proposed that parasitoids would have strong responses to these synomones if the parasitoid was a specialist on one host species found on a single plant species, or parasitoids that can utilize multiple hosts on a single plant species. In these cases, herbivore-induced synomones of the PHC are not only detectable over a long distance but also reliable indicators of host presence.

The ability for parasitoids wasps to utilize volatile compounds has been well established, but most studies have either focused on specialists (Colazza, McElfresh, & Millar, 2004; de Moraes, Lewis, Pare, Alborn, & Tumlinson, 1998; Du, Poppy, Powell, & Wadhams, 1997; Gols, Bullock, Dicke, Bukovinszky, & Harvey, 2011; McCall, Turlings, Lewis, & Tumlinson, 1993; Röse, Lewis, & Tumlinson, 1998; Tumlinson, Turlings, & Lewis, 1993; Xiu et al., 2019), or on a single host-flexible species (D'Alessandro, Brunner, von Mérey, & Turlings, 2009; Ponzio et al., 2016; Röse et al., 1998; Tumlinson, Lewis, et al., 1993; Turlings, Tumlinson, & Lewis, 1990; Wei et al., 2007) and no other related species. Although Vet and Dicke's (1992) hypotheses may hold true for parasitoids that specialize on a single host species, it is likely that host-flexible parasitoids utilize these cues differently. An overarching issue with each one of these hypotheses is that they approach the system from a bottom-up perspective, describing infochemical use between the third and second trophic level (parasitoid and host herbivore), and how interactions between the second and first (host herbivore and plant) could impact infochemical use by the third. In doing so, these hypotheses downplay the potential for infochemical cues from the first trophic level, outside of herbivore-induced infochemicals, to function as reliable indicators for the location of a suitable host herbivore. In this study we examine how infochemicals from the plant impact host selection in parasitoids, and whether closely related host-flexible parasitoids exhibit a differential preference for different plant volatile profiles.

An excellent group for testing this question is *Eadya* Huddleston & Short, 1978 (Hymenoptera: Braconidae: Euphorinae), a small genus of parasitoid wasps endemic to Australia. These wasps attack paropsine leaf-beetles (Coleoptera: Chyrsomelidae: Chrysomelinae) that feed on*Eucalyptus* L'Héritier, 1789 (Myrtales: Myrtaceae). *Eadya*is currently comprised of 6 described species (Huddleston & Short, 1978; Ridenbaugh, Barbeau, & Sharanowski, 2018), all of which are host flexible to varying degrees. Species such as *Eadya*

*daenerys*Ridenbaugh, 2018 and *Eadya spitzer* Ridenbaugh, 2018 were documented from four different hosts, while *E. paropsidis*Huddleston & Short, 1978 has been documented on three hosts between Tasmania and mainland Australia, but only reared from *Paropsis tasmanica* Baly, 1866 in Tasmania (Peixoto et al., 2018).

Species of *Eadya* are also of interest as biological control agents targeting invasive paropsine beetles infesting *Eucalyptus*plantations in New Zealand, with *E. daenerys* approved to control*P. charybdis* Stål, 1860 (T. M. Withers, Allen, Todoroki, Pugh, & Gresham, 2020) and *E. annleckieae* Ridenbaugh, 2018 as a potential agent to control *Paropsisterna* (=*Pst. variicollis*(Chapuis) in Peixoto et al., 2018) (Nahrung et al., 2020). Leschen et al. (2020) recently synonymized *Pst. variicollis* under *Pst. cloelia* (Stål) but referenced no type material.

Given the host flexibility observed within *Eadya* , plant infochemicals may play a more important role than host cues in host selection. To assess this, we perform cophylogenetic analyses to test for evidence of cospeciation between *Eadya* and their hosts. If the parasitoids show strong evidence of cospeciation with their hosts, we expect that host infochemicals would have a strong impact on host location and selection. To examine how infochemicals from the plant potentially impact host location and selection in closely related host-flexible parasitoids, we test for distinct chemoprofiles in*Eucalyptus* species and test whether these profiles are different for damaged versus undamaged leaves, suggesting an herbivore-induced response. Then we ask if these plant chemoprofiles are good predictors of parasitoid and/or host species, which would indicate that plants have a strong influence on host location and selection in the parasitoid.

Materials and Methods

Sample Collection

Samples were collected between October 31st - December 10th, 2018 from *Eucalyptus globulus* Labill. plantations around Hamilton, Victoria, Australia, owned and operated by Australian Bluegum Plantations (*http://www.austgum.com.au/*) (Fig. 1) (SI Table 1 & 2). Plantations were chosen to ensure efficient collecting. Another species of *Eucalyptus* was present as volunteer plants but could not be identified to species due to an absence of reproductive parts (*Eucalyptus* sp. 1). Beetle larvae between the first and fourth instar were collected by hand and reared gregariously in 750mL plastic takeaway containers, with all beetles collected from a single tree reared in the same takeaway container. Before pupation, beetle larvae were placed into 2cm x 2cm nylon pouches, stapled shut, and isolated in small vials in anticipation of*Eadya* emergence to establish definitive host-parasitoid associations following Davy et al. (2016). A total of 98 parasitoids were reared from the beetle larvae.

For each beetle and parasitoid collected the GPS coordinates of the tree were recorded. Upon reaching the final instar, the beetle host was isolated before pupation when the parasitoid emerges to establish a definitive parasitoid-host association. Upon emergence of a parasitoid, what was left of the beetle host was immediately stored in 95% ethanol. Parasitoid wasp larvae that emerged and successfully spun cocoons were kept for 50 days, allowing for adult morphological characters to develop, and then preserved in 95% ethanol. Those that failed to exit the host or successfully spin a cocoon were immediately preserved in 95% ethanol. For each beetle larva collection event, three leaf samples were also collected: an herbivore damaged leaf, an undamaged leaf of the same age class as the damaged leaf (either flush (newly expanding) or fully expanded glaucous foliage), and an undamaged leaf of the other age class. Leaves were labeled accordingly and dried in individual plastic sandwich bags with silica desiccating powder.

DNA Extraction and PCR Protocol

Genomic DNA extractions were performed using the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's protocol. DNA was extracted from Immature beetles and parasitoids via destructive sampling, while DNA from adult parasitoids were extracted non-destructively from the right midleg as the extraction medium with the remainder of the wasp retained as a voucher and deposited at the University of Central Florida Collection of Arthropods (UCFC). All specimens for which DNA was extracted were assigned a unique voucher code consisting of the letters RDR followed by three numbers (e.g. RDR001). For

the identification of destructively sampled specimens, the barcode region of Cytochrome oxidase c subunit 1 (*CO1*) was amplified using universal primers (Forward: 5'- GGT CAA CAA ATC ATA AAG ATA TTG G - 3'; Reverse: 5' - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3') (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). Polymerase chain reactions were performed using 1 µL of template DNA, 0.2 mM dNTP solution (New England Biolabs, (NEB)), 4 mM $MgSO_4$, 1X standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2) (NEB), 400 nM of each primer (Integrated DNA Technologies), 1 unit of Taq DNA polymerase (NEB), and PCR grade water to bring the reaction to a volume of 25 µL. Thermocycler settings were as follows: initial denaturation at 95°C for 1 minute, 34 cycles of denaturation at 95°C for 15 seconds, annealing at 49°C for 15 seconds, and extension at 72°C for 45 seconds. PCR products were visualized using 5 µL of PCR product mixed with 1 µL of dye, loaded into a 1% agarose gel, and imaged after separation via gel electrophoresis. Samples observed with faint bands were re-run using 2 µL of template DNA and 1.25 units of *Taq* DNA polymerase (NEB) for 38 cycles. PCR products were processed using magnetic bead clean-up and sequenced on an Applied Biosystems 3730x 96 capillary sequencer at the UK Healthcare Genomic Core Laboratory. Forward and reverse reads were trimmed, assembled into contigs, and then edited for quality using Geneious Prime 2020.04 (*https://www.geneious.com*). Sequences were uploaded to GenBank under accession numbers MT246305-MT246448.

Phytochemical Extraction and Analysis

Desiccated leaves were homogenized under liquid nitrogen and stored at -80°C prior to extraction. To extract a broad range of polar and nonpolar metabolites, the homogenized samples were mixed with 1 mL of a 1:1 methanol-chloroform solution in conjunction with 1 µL of ethyl-decanoate (Sigma-Aldrich, Inc.) as an internal standard. Sample extracts were vortexed in an orbital shaker for 2 hours to expedite extraction at 160 rotations min^{-1}. Post extraction, each sample was filtered through a 0.2 µm nylon filter into a 2 mL amber glass vial, where 1 µL of the sample extract was used for direct injection into a single quadrupole GCMS-QP2020 NX gas chromatograph-mass spectrometer (Shimadzu, Inc.). Each extract was injected at 230°C in splitless mode with helium carrier gas flow set to 2 mL min^{-1} by an AOC-6000 autosampler (Shimadzu, INC.). The oven was held isothermal at 40°C for 1 min, ramped at 15°C min^{-1} to 330°C where it was held isothermal for 1 min. Electron impact mass spectra were recorded in full scan mode from m/z 50 to 450 in the single quadrupole mass spectrometer. Data were initially preprocessed using GCMS solutions v1.4 (Shimadzu, Inc.). Signals were integrated using total ion count and measurements such as the area and height of chromatogram peaks were normalized to the internal standard. Compounds were putatively identified by comparing observed mass spectra with the NIST mass spectra library using a 75% significance index cutoff.

Phylogenetic Analyses and Species Delimitation

CO1 sequences were aligned by hand using the reading frame for reference in BioEdit v 7.2.5 (Hall, 1999) as there were no indels across sequences. Each sequence was evaluated for evidence of amplification of a nuclear mitochondrial insertion (NUMT) (Lopez, Yuhki, Masuda, Modi, & O'Brien, 1994) based on the criteria outlined by Zhang and Hewitt (1996). Sequences suspected of being NUMTs were removed from future analysis. Maximum likelihood and Bayesian phylogenetic analyses were performed to identify specimens destructively sampled using IQTree v 1.6.10 (Nguyen, Schmidt, von Haeseler, & Minh, 2014) and MrBayes v 3.2.7 (Ronquist et al., 2012) on the CIPRES Science Gateway v 3.3 (Miller, Pfeiffer, & Schwartz, 2010). *Afrocampsis* sp. (Hymenoptera: Braconidae: Helconinae) and *Johannica gemellata*(Coleoptera: Chrysomelidae: Chrysomelinae) were used as outgroups for the *Eadya* and host beetle phylogenies, respectively. Confidently identified voucher sequences of both parasitoids and beetles from Peixoto et al. (2018) and Nahrung et al. (2020) were included in each analysis to confirm the species of parasitoids and beetle hosts that were destructively sampled. In addition, four *Eadya* specimens collected in Tasmania and sent to the first author for identification were included (RDR129 - RDR132). One specimen (RDR237) was removed from the wasp dataset prior to phylogenetic analyses as a nucleotide BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) search (megablast under default settings) of the nr nucleotide NCBI database indicated greatest similarity to unrelated species of *Maxfischeria* (Braconidae: Maxfischeriinae) (Boring, Sharanowski, &

Sharkey, 2011). Two specimens were removed from the host dataset prior to phylogenetic analyses. RDR274 was most similar to species of *Eadya* based on a BLAST search (same as above), indicting the host sample was contaminated by the parasitoid. RDR301 contained a stop codon in the coding sequence, indicating a NUMT was potentially amplified.

The best fitting model of evolution was determined for each alignment based on Bayesian Information Criterion (BIC) using ModelFinder (Kalyaanamoorthy, Minh, Wong, von Haeseler, & Jermiin, 2017) within IQTree v 1.6.10 on the CIPRES Science Gateway v 3.3. For the wasp (*Eadya*) dataset, the top performing model was K3Pu (BIC = 5986.3377) with parameters for empirical base frequencies (+F) and a gamma distribution of rate heterogeneity (+G). As this model is not supported within MrBayes, HKY+F+G (BIC = 5988.4978) was used as its BIC score was within Δ5 of the top performing model. For the beetle dataset, the top performing model was HKY+F+G (BIC = 6554.0634). The maximum likelihood analyses were performed with 1000 ultrafast bootstraps (Hoang, Chernomor, Von Haeseler, Minh, & Vinh, 2017), while the Bayesian analyses consisted of two independent runs with four chains each, for a total of 15,000,000 generations, sampling every 1000 generations with a 25% burn-in applied. Convergence of the two independent runs was evaluated using the average standard deviation of split frequencies, the potential scale reduction factor, and minimum estimated sample size output by MrBayes. Additionally, parasitoid and beetle Bayesian phylogenies were constructed using a dataset supplemented with Tasmanian specimens from Peixoto et al. (2018) for use in the Procrustes Approach to Cophylogeny (PACo) analysis outlined below. These phylogenies were constructed using the same methods listed above, using a HKY+F+G (BIC = 7587.3901) for the parasitoid dataset and HKY+F+G with a parameter for invariant sites (+I) for the beetle dataset (BIC = 8949.7961; Δ5.8 from top model). The intra- and inter-specific genetic distances were calculated for each clade recovered in the phylogenetic analyses using MEGA v 7.0 (Kumar, Stecher, & Tamura, 2016) with the Kimura 2-parameter model of molecular evolution (Kimura, 1980). The maximum likelihood and Bayesian trees were visualized using FigTree v 1.4.3 (Rambaut, 2012), and edited using Adobe Illustrator Creative Cloud (Adobe Systems Inc.). Alignment files can be found on Figshare (*www.figshare.com* DOI: 10.6084/m9.figshare.17105768).

Parasitoid-Host Cophylogenetic Analyses

When cospeciation between parasites and their hosts occurs, we expect congruence between parasitoid and host phylogenies and a 1:1 relationship between the interacting species, demonstrating strong host specialization (Balbuena, Míguez-Lozano, & Blasco-Costa, 2013; Fahrenholz, 1913; Legendre, Desdevises, & Bazin, 2002; Page, 2003). Given the degree of host flexibility observed within *Eadya*parasitoids (Peixoto et al., 2018; Ridenbaugh et al., 2018), cospeciation with their hosts was not expected. To test this, two cophylogenetic methods were used, an event-based and distance-based method. The former compares parasitoid and beetle tree topologies and their associations to reconstruct cospeciation, duplication, host switch, losses, and failure-to-diverge events, while the later uses distance matrices produced from the parasitoid and beetle phylogenies and their associations to statistically test for congruences between the two topologies. In the event-based reconstruction cospeciation events occur when the parasite speciates in unison with the host. Duplication events are when speciation in the parasite occurs independently from the host. Host switching is the result of a duplication event in which the parasite jumps to a new host lineage. Loss and failure-to-diverge events are very similar in that both occur when the host speciates, but the parasite does not. However, in loss events the parasite only remains on one of the new host lineages, and in failure-to-diverge events the parasite remains on both host lineages. For the event-based analysis Jane 4 (Conow, Fielder, Ovadia, & Libeskind-Hadas, 2010) was used, with the genetic algorithm set to run for 200 generations with a population size of 5000 using two different cost models. The first model was the default cost model for Jane, with cospeciation set to 0, duplication and host switching set to 2, and all other event types set to 1. An alternative model was used with cospeciation set to -10 and all other events set to default to test the algorithms sensitivity to the cost settings. For this method the input was trees drawn by hand using the tree editor within Jane 4 and with clades collapsed for each species to control for the potential overestimation of cospeciation events due to sampling bias (Bass, 2019). For the distance-based method, a PACo analysis (Balbuena et al., 2013) was used as implemented in R (R Core Team, 2016) using the R package "PACo" (Hutchinson, Cagua, Balbuena,

Stouffer, & Poisot, 2017). PACo was run for 10,000 permutations with the r0 randomization algorithm from the R package "vegan" (Oksanen et al., 2016).

Principal Component Analysis

To examine the association between plant infochemicals and host location and selection in closely related host-flexible parasitoids a principal component analysis was performed using the relative area of the chromatogram peaks, a measurement of abundance for each compound, obtained from the GC/MS and parasitoid-host association data. Four separate principal component analyses were run in R using the package "FactoMineR" (Lê, Josse, & Husson, 2008) and visualized using the package "Factoextra" (Kassambara & Mundt, 2017). The first principal component analysis included all *Eucalyptus* leaves collected to examine differences in phytochemistry between the two species of*Eucalyptus* sampled (*globulus* vs. sp. 1). The second principal component analysis included damaged and undamaged flush*Euc. globulus* leaves(the age class preferred by the beetles) to explore variation in plant chemoprofiles when damaged, suggesting a specific herbivore-induced response. Only *Euc . globulus*were examined because there were not enough samples to adequately assess herbivore-induced responses in the other *Eucalyptus* species. The third principal component analysis included damaged leaves corresponding to unparasitized beetles, with beetles identified by their distinctive larval coloration: yellow body with a prominent black dorsal strip in*Paropsisterna cloelia* ; and black body with orange/yellow lateral stripes in *Pst. agricola* . This subset of samples permitted examination of the relationships between infochemicals and the observed herbivore assemblage. The fourth principal component analysis included damaged leaves corresponding to parasitized beetles from which different species of *Eadya* were reared, in order to examine the relationship between infochemicals from the plant and *Eadya* host selection To minimize the effects of pseudo-replication in the principal component analysis in the parasitoids and beetles, the dataset was collapsed to only include one sample per *Eadya* species for each tree. To confirm separation in each of the four principal component analyses, one-way ANOVAs were performed using the first and second principal components, with Tukey post-hoc tests for the parasitoids and beetle analyses. To determine phytochemical compounds which may be utilized as infochemicals by *Eadya* , compounds were ranked in descending order by the absolute value of PC1 – PC2 variable loadings. The R script for the PCA and ANOVAs, as well as all data files, can be found on Figshare (*www.figshare.com*10.6084/m9.figshare.17105768).

Results

Phylogenetic Analyses and Species Identification

A total of 67 parasitoids and 72 beetle hosts were successfully sequenced, and of these, there were 51 pairs of parasitoids plus their corresponding beetle hosts that were both successfully sequenced. The final alignments included 80 sequences for the parasitoid alignment, including one outgroup and 8 voucher sequences (Peixoto et al., 2018), and 85 sequences for the host beetle alignment, including one outgroup and 12 voucher specimens (Nahrung et al., 2020; Peixoto et al., 2018). Both alignments consisted of 684 characters.

For the parasitoids, both Bayesian (Fig. 2) and maximum likelihood phylogenetic analyses (SI Fig. 1) recovered five distinct and well supported clades (pp = 1; Bootstrap [?] 90). Four of these clades corresponded to the Peixoto et al. (2018) voucher specimens. One clade (hereafter referred to as *Eadya* sp. 1) did not fall with any known vouchers and all three specimens in the clade did not have adult vouchers for identification. As the specimens could be *E. duncan*Ridenbaugh, 2018 or *Eadya falcata* Huddleston & Short, 1978 which have never been sequenced, or a new yet to be described species, the identity of the parasitoids could not be confirmed. All *Eadya*specimens sequenced for this study were recovered in either the *E. daenerys* , *E. annleckieae* , or *E.* sp. 1 clades. The only difference between the Bayesian (Fig. 2) and maximum likelihood (SI Fig. 1) phylogenies was the placement of the *E.* sp. 1 clade. The Bayesian analysis recovered *E.* sp. 1 sister to *E. daenerys*with a posterior probability of 0.90, while the maximum likelihood analysis recovered it sister to the clade of *E. paropsidis* +*E. spitzer* with poor support (Bootstrap = 58).

For the beetles, both Bayesian (Fig. 3) and maximum likelihood (SI Fig. 2) analyses recovered six distinct and strongly supported clades (pp = 1; bootstrap [?] 91) with identical relationships. All recovered beetle clades corresponded to UCF voucher specimens from Peixoto et al. (2018) and Nahrung et al. (2020). Host

specimens sequenced for this study were recovered in four out of the six beetle species for which we had vouchers: *Paropsis aegrota-elliotti* Selman, 1983;*Paropsisterna m-fuscum* (Boheman, 1859); *Pst .agricola* (Chapuis, 1877); and *Pst . cloelia* clades. A distinct barcode gap, with interspecific distances greater than intraspecific distances, was observed in both the parasitoid (Table 1) and beetle datasets (Table 2). The largest intraspecific genetic distance for parasitoids was 1.06% in *E. spitzer* and 0.78% in*Pst. m-fuscum* for the beetle hosts. Interspecific genetic distances in parasitoids ranged from 6.45% between *E. daenerys*and *E.* sp. 1, to 31.07% between *E. paropsidis* and*E. annleckieae* (Table 1). Large values were also recovered in the host dataset, with the smallest interspecific distance being 9.77% between *Pst. agricola* and *Pst. variicollis** , and the largest 19.07% between *P. charybdis* and *Pst. agricola* (Table 2).

Given that all sequenced specimens fell within well supported clades with distinct barcoding gaps, we could identify the unknown sequenced specimens for both parasitoids and hosts as follows: 20 *E. daenerys* , 44 *E. annleckieae* (not including the four Tasmanian specimens), three *E.* sp. 1, one *P. aegrota elliotti* , five*Pst. m-fuscum* , 17 *Pst. agricola* , and 49 *Pst. cloelia* . These identifications were used for all downstream analyses.

Parasitoid-Host Cophylogenetic Analyses

The relationships of the parasitoid and beetle Bayesian phylogenies used in the cophylogenetic analyses were identical to those recovered in this study and Peixoto et al. (2018) (SI Fig. 3 & 4). Using the default cost model, ten isomorphic solutions were recovered (Fig. 4) with zero cospeciation, three duplication, one duplication and host switch, 13 losses, and six failure-to-diverge events for a total cost of 24. The second Jane analysis using an alternative cost model (cospeciation set to -10, a much lower cost than all other events) were identical to the default cost model (SI Fig. 5) with ten isomorphic solutions as listed above and a total cost of 24. These results indicate that duplication, and not cospeciation, is the dominate speciation event and that the lack of cospeciation events inferred are robust.

The PACo distance-based analyses returned a sum of squares of 288.55 with p-value < 0.001 showing strong phylogenetic congruence. This is an indication of cospeciation between the parasitoid and host phylogenies, which contradicts the results of the Jane analyses. The Procrustes residuals indicated that associations between *E. annleckieae* and *Pst. cloelia* had a large amount of cophylogenetic signal (residuals closer to 0) compared to all other species of *Eadya* . Separating these associations from the rest and comparing the residuals using a Welch's t-test demonstrated that the cophylogenetic signal between *E. annleckieae* and *Pst. cloelia* is significantly greater than all other associations ($t = -6.48$, $df = 73.35$, $p < 0.001$). This indicates that the cophylogenetic signal observed between the parasitoids and their beetle hosts is largely contained in associations between *E. annleckieae*and *Pst. cloelia* , signifying sampling bias.

Principle Component Analysis

Thirty leaves were successfully extracted with 112 compounds identified (Table. 7), with 11 leaves of *Euc.* sp. 1 and 19 leaves of*Euc. globulus* . Of the 51 parasitoid -beetle pairs, damaged leaves were successfully extracted for 49 pairs. The first PCA examined all*Eucalyptus* leaves to see if there were distinct chemoprofiles across the two species: 54.1% of the variation was explained in the first five principal components (PCs), with 21.4% in the first, and 10.7% in the second. Separation of the two *Eucalyptus* species was observed in PC1 ($F_{1,28} = 24.09$, $P < 0.001$) but not PC2 ($F_{1,28} = 3.633$, $P = 0.067$) (Fig. 5A; Table 3). One *Euc.* sp. 1 sample grouped with the *Euc. globulus*specimens (VIC083 MD). This *Euc.* sp. 1 leaf was the only mature herbivore damaged leaf collected. This suggests that there are distinct chemoprofiles between the two species of *Eucalyptus* tested in this study.

For the second PCA including flush, damaged and undamaged *Euc. globulus* leaves, 71.1% of the variation was explained in the first 5 principal components, with 25.9% in the first, and 15.3% in the second. Clear separation between the two groups was not observed in either PC1 ($F_{1,9} = 1.3$, $P = 0.284$) or PC2 ($F_{1,9} = 1.513$, $P = 0.25$) (Fig. 5B; Table 3).

For the third PCA including leaves damaged by the four species of beetles, 75.3% of the explained variation

was observed in the first five PCs, with 31.3% in the first, and 14.1% in the second. Clear separation is observed in PC2 ($F_{3,9} = 26.1$, $P < 0.001$) but not in PC1 ($F_{3,90} = 2.266$, $P = 0.0862$) (Fig. 5C; Table 3). Leaves damaged by *Pst. cloelia* were found to be significantly separated from those damaged by *Pst. agricola* ($P < 0.001$) and *Pst. m-fuscum* ($P < 0.001$) in the Tukeys Post Hoc analysis of PC2 (Table 4). These results suggest that *Pst. cloelia* prefer different plant chemoprofiles to the other species.

For the fourth PCA examining leaves damaged by beetles from which*Eadya* parasitoids were reared, 83% of the explained variation was observed in the first five PCs, with 30.6% in the first, and 16.5% in the second. Separation is observed in PC1 ($F_{2,46} = 7.611$, $P = 0.00139$), and PC2 ($F_{2,46} = 4.183$, $P = 0.0214$) (Fig. 5D; Table 3). Of the three species only *E. daenerys* and*E. annleckieae* were significantly separated ($P > 0.0037$), and only for PC1 (Table 4). These results suggest that, like the beetles, species of *Eadya* are attracted to species-specific chemoprofiles of *Eucalyptus* .

Discussion

Infochemical cues play an important and crucial role in parasitoid host location and selection (Tumlinson, Lewis, et al., 1993; Vet & Dicke, 1992; Vinson, 1976). Vet and Dicke (1992) framed the use of these cues by parasitoids as a tradeoff between reliability and detectability, which they called the reliability-detectability problem. Host infochemical cues were hypothesized to be a highly reliable indicator of a host's presence, but due to their localized nature would not be detectable over long distances. Conversely, infochemical cues from the plant were hypothesized to be highly detectable but poor indicators of host presence and thus unreliable, unless they were induced by herbivory. Vet and Dicke (1992) also hypothesized that when a parasitoid that had one or most hosts that fed on a single plant species, plant infochemicals could become both reliable and detectable. In a third scenario in which the parasitoid specializes on a host species that feeds upon multiple plant species, host infochemical cues were hypothesized to be highly reliable while herbivore induced synomones were not. Here, however, the synomones from each plant species could be learned and associated with the host, thus compensating for the low detectability of the host kairomones. Finally in a fourth scenario , in which both the parasitoid and its hosts were extreme generalists, Vet and Dicke (1992) hypothesized that infochemical cues would not be used. Although Vet and Dicke (1992) acknowledged each of these four scenarios were extremes on a continuum, and that intermediates could be found in a nature, they did not specifically discuss cases that involved oligophagous parasitoids and their hosts. This is the situation for*Eadya* parasitoids, which attack multiple related beetle hosts that feed on multiple related *Eucalyptus* plants. Thus, the question remains as to how these parasitoids might use infochemicals to locate their hosts.

For parasitoids that specialize on one host and rely heavily on host infochemical cues, we would expect to see some degree of coevolution leading to cospeciation between parasitoids and their hosts (Page, 2003). As *Eadya* are host-flexible (Peixoto et al., 2018), there would be less expectation of coevolution and cospeciation with their beetle hosts. Indeed, our data did not support cospeciation in the event-based analysis, but rather provided robust evidence for duplication and host-switching with duplication being the dominant type of evolutionary event. Although the distance-based analysis demonstrated strong congruence between parasitoid and beetle phylogenies indicating cospeciation, further exploration of the data suggested that cophylogenetic signal was restricted to the interactions between*E. annleckieae* and *Pst. cloelia* (Hutchinson et al., 2017). This large disparity in cophylogentic signal between *E. annleckieae* and the rest of *Eadya* can be attributed to sampling error. Peixoto et al. (2018) reared *E. annleckieae* from *P. charybdis, Pst. cloelia, Pst. selmani,* and *Pst. nobilitata*(Erichson, 1842) but DNA was only successfully extracted for *Pst. cloelia.* As a phylogram is required to calculate the distance matrices for the PACo distance-based analysis, only *Pst. cloelia* could be included, creating a false one-to-one relationship between *E. annleckieae* and *Pst. cloelia* . Thus, *E. annleckieae* is the only parasitoid species with a single host association in the distance-based analysis, which most likely biased the PACo analysis. Although we cannot rule out the use of host infochemical cues in*Eadya* as these were not directly tested here, the lack of evidence for cospeciation between these parasitoids and their beetle hosts as well as their known host flexibility, suggests these cues are unlikely to be the dominant driver in the location and selection of a suitable host.

Although Vet and Dicke (1992) discussed plant synomones and hypothesized how the compounds are used

in a tritrophic context, this was only considered from a bottom-up perspective in which the herbivore host breadth solely influences whether plant infochemicals could reliably be used by the parasitoid to locate its herbivore host. For parasitoids that specialize on a single host, the low reliability of plant cues discussed in Vet and Dicke (1992) likely holds true, as in most cases these signals cannot guarantee the presence of a suitable host unless they are herbivore-induced volatiles (de Moraes et al., 1998). However, this may not be the case for less specialized parasitoids that can survive on a wider range of hosts, which in turn utilize a range of closely related plant species. Here, plant infochemicals, even if not herbivore-induced, may be both reliable and detectable for parasitoids if they specialize on a single host tree species, or a distinct infochemical signature, as long as they can utilize multiple hosts associated with that species/infochemical signature. This may allow parasitoids to reduce competition with related parasitoid species through plant specialization rather than host specialization. Here we find evidence that *Eucalyptus* infochemicals are species-specific, corresponding to specific species of *Eadya* and their beetle hosts (Fig. 5A & D). However, since we found no evidence of cospeciation between the parasitoids and their hosts we suggest that host selection in *Eadya* is influenced by *Eucalyptus* phytochemistry irrespective of the specific beetle host used. Although, several limitations to our study restrict us from considering this conclusion definitive. 1) *Euc. globulus* was the intended target plant of this study, with *Euc.* sp. 1 being a volunteer in a single plantation, and therefore was not widely sampled. 2) Sampling was limited to a small area in western Victoria and does not represent the endemic range of *Euc. globulus* nor that of the beetle or parasitoid species. 3) As only juvenile *Euc. globulus* plantations were targeted, the full range of *Eucalyptus* utilized by*Eadya* is still unknown.

We identified a diverse array of plant metabolites that could accurately distinguish between the two groups of *Eadya* (Figure 5D, Table S3). Among top contributing compounds to axes 1 & 2, we identified two general groups of compounds: (1) fatty acid derivatives (Docosanoic acid, phenylmethyl ester (C106), 4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-(4-methoxyphenyl)-6,8-dimethyl- (C105), and Octadecanoic acid, 2,3-dihydroxypropyl ester) and (2) compounds with known larvicide activity (agarospirol, ascaridole epoxide, alpha-pinene, alpha-phellandrene, phytol, and eucalyptol) (de Castro et al., 2016; Johnson & Singh, 2017; Kaczmarek, Wrońska, Kazek, & Boguś, 2020; Mohamed & Jong, 2014)

Docosanoic acid has the potential to function as either a kairomone or synomone in both insects and plants. Docosanoic acid is a derivative of*Eucalyptus* cuticular wax, a known Coleopteran attractant, and changes in Docosanoic acid correspond with larval maturation in certain insects (Courtney, Lassak, & Speirs, 1983; Gosney et al., 2016; Kaczmarek et al., 2020; Sarkar & Barik, 2015). Docosanoic acid is commercially manufactured and presents as a strong candidate for attractants for *Eadya* in New Zealand *P. charybdis*biological control efforts (T. Withers, Todoroki, Allen, Pugh, & Gresham, 2019) with low potential for toxic effects on non-target insects (Kaczmarek et al., 2020; Sarkar & Barik, 2015). Although we can discern which *Eucalyptus* infochemicals are associated with host use and that compounds can separate the two groups of *Eadya* , future work should aim at directly testing for attractant responses across the genus *Eadya* . Further work to identify compounds that attract host flexible parasitoids should consider volatile compounds that are present in both the host and associated plant species, with foci on fatty acid derivatives based on this study's results.

We did not find evidence for an herbivore-induced response, as no overall pattern could be discerned between herbivore damaged and undamaged *E. globulus* leaves from the PCA (Fig. 5B). However, these inconclusive results may be due to the methodology of our study. As herbivore damaged leaves were collected from the field, it was not possible to determine when the leaves were damaged. The production of volatile compounds is metabolically expensive (Cipollini, Walters, & Voelckel, 2018) and is likely not maintained indefinitely after herbivore damage. Based on our data, we were unable to determine whether herbivore damage induced the production of volatile compounds in*Euc. globulus* , but cannot rule out the possibility until a study can be undertaken using methodology better suited for this question (Materić et al., 2015).

With this evidence we propose an amendment to Vet and Dicke's (1992) reliability-detectability hypothesis to include how infochemicals are used when oligophagous parasitoids utilize oligophagous hosts. In this

ecological context, we expect little to no response to host kairomones and no evidence of cospeciation between the parasitoid and their hosts, but a strong response to plant synomones and potentially herbivore-induced plant synomones. Although host kairomones are the most reliable indicators of host presence, especially for specialists, plant synonomes, whether induced or not, may play a larger role for oligophagous parasitoids. The ability to successfully utilize multiple hosts is an adaptive advantage in instances when hosts have a patchy distribution or small population size, or when multiple parasitoids are competing for a limited number of hosts (Price, 1971). Further, if hosts feed on multiple plants, specializing on one plant where multiple plant species are present may limit competition between parastioids.*Eadya annleckieae* and *E. daenerys* ' wide niche breadth and preference for hosts that feed on plants with different chemoprofiles may aid in reducing competition between the two species. This study greatly expands our understanding of the complex interactions within the tri-trophic *Eadya* system and how they utilize plant infochemicals for host location and potentially to limit intra-specific competition. To further understand these interactions a more robust taxon sampling of*Eucalyptus* and *Eadya* across their native range is required to definitively establish the adaptive advantages of host flexibility and the use of plant synomones for host location in similar tri-trophic systems. Subsequently no-choice competition assays between *Eadya*species using distinct infochemical signatures should be conducted under a controlled environment to establish the competitive advantages of plant specialization.

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Author Contributions

RDR designed the study, collected samples in the field and reared the parasitoids, generated the molecular data, analyzed the molecular and phytochemical data, and led the writing. JAD generated the phytochemical data and assisted with the phytochemical analyses. MH collected samples in the field and assisted with the rearing of the parasitoids. CMM assisted with the design of the study and the phytochemical analyses. TMW assisted with the design of the study and funding. BJS assisted with the design and analysis of the study and writing.

Data Accessibility Statement

All sequences generated for this study have been uploaded to GenBank under accession numbers MT246305-MT246448. All alignment, R code, and input files have been uploaded to FigShare (www.figshare.com DOI: 10.6084/m9.figshare.17105768).

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Figures and Tables

Figure 1. Map of collecting locations in Victoria, Australia. This map was generated using the R packages "ggmap" (Kahle & Wickham, 2013) and "ggplot2" (Wickham, 2016).

Figure 2. *Eadya* Bayesian *CO1* Phylogeny. For all relevant nodes the posterior probabilities are listed. The scale bar refers to the number of substitutions for tree branches.

Figure 3. Host Bayesian *CO1* Phylogeny. For all relevant nodes the posterior probabilities are listed. The scale bar refers to the number of substitutions per site for tree branches.

Figure 4. Jane 4 Default Cost Co-phylogeny. Cospeciation events are indicated by a white circle. Duplication events are indicated by a solid-colored circle. Duplication and Host Switch events are indicated by a solid-colored circle and an arrow. Loss events are indicated by a dashed line. Failure to Diverge events are indicated by a squiggly line.

Figure 5A-D. Principal Component Analysis Plots. A) Plot of first and second component comparing *Eucalyptus* species using both damaged and undamaged leaves. B) Plot of first and second component comparing*Eucalyptus globulus* damaged and undamaged leaves. C) Plot of first and second component comparing beetle species using damaged*Eucalyptus* leaves. D) Plot of the first and second component comparing *Eadya* species using damaged *Eucalyptus* leaves.

Table 1. *Eadya* Intra and Interspecific Distance Matrix for*CO1* . Intraspecific distance is highlighted in grey.

Table 2. Host Intra and Interspecific Distance Matrix for *CO1* . Intraspecific distance is highlighted in grey.

Table 3. Results of the ANOVAs for the Principal Component Analyses visualized in Figure 5A-D.

Table 4. Results of the Tukeys Post Hoc Analyses for the Parasitoid and Beetle ANOVAs

Supporting Information

SI Figure 1. *Eadya* Maximum Likelihood Tree. For all relevant nodes the bootstrap values are listed. The scale bar refers to the number of substitutions for tree branches.

SI Figure 2. Host Beetle Maximum Likelihood Tree. For all relevant nodes the bootstrap values are listed. The scale bar refers to the number of substitutions for tree branches.

SI Figure 3. PACo *Eadya* Bayesian Tree. For all relevant nodes the posterior probabilities are listed. The scale bar refers to the number of substitutions for tree branches.

SI Figure 4. PACo Host Beetle Bayesian Tree. For all relevant nodes the posterior probabilities are listed. The scale bar refers to the number of substitutions for tree branches.

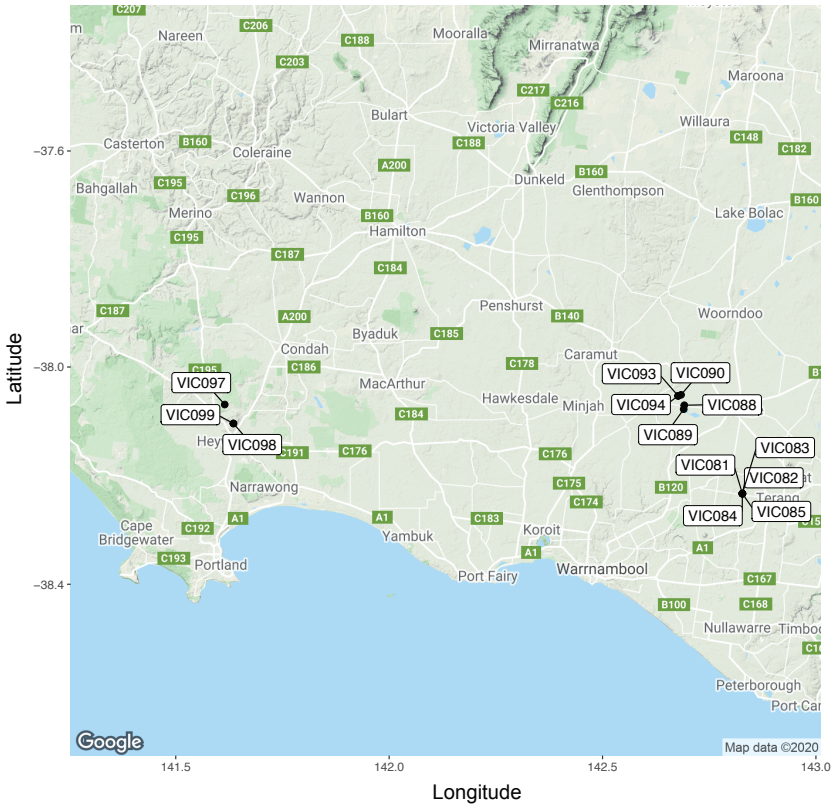
SI Figure 5. Jane4 Alternative Co-Phylogeny. Cospeciation events are indicated by a white circle. Duplication events are indicated by a solid-colored circle. Duplication and Host Switch events are indicated by a solid-colored circle and an arrow. Loss events are indicated by a dashed line. Failure to Diverge events are indicated by a squiggly line.

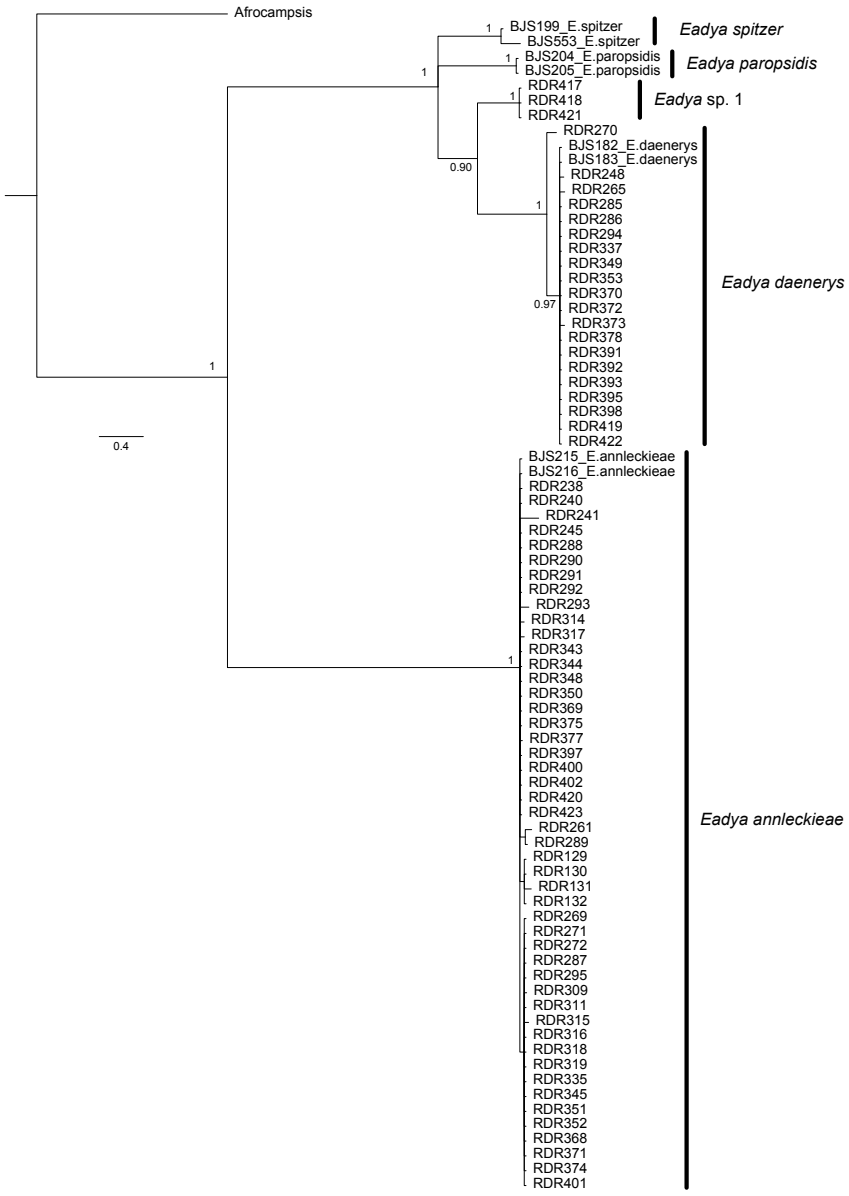
SI Table 1. List of all *Eadya* material examined along with their DNA voucher number, DNA voucher numbers of the host it was reared from, and the unique identifier of the tree the host was collected from.

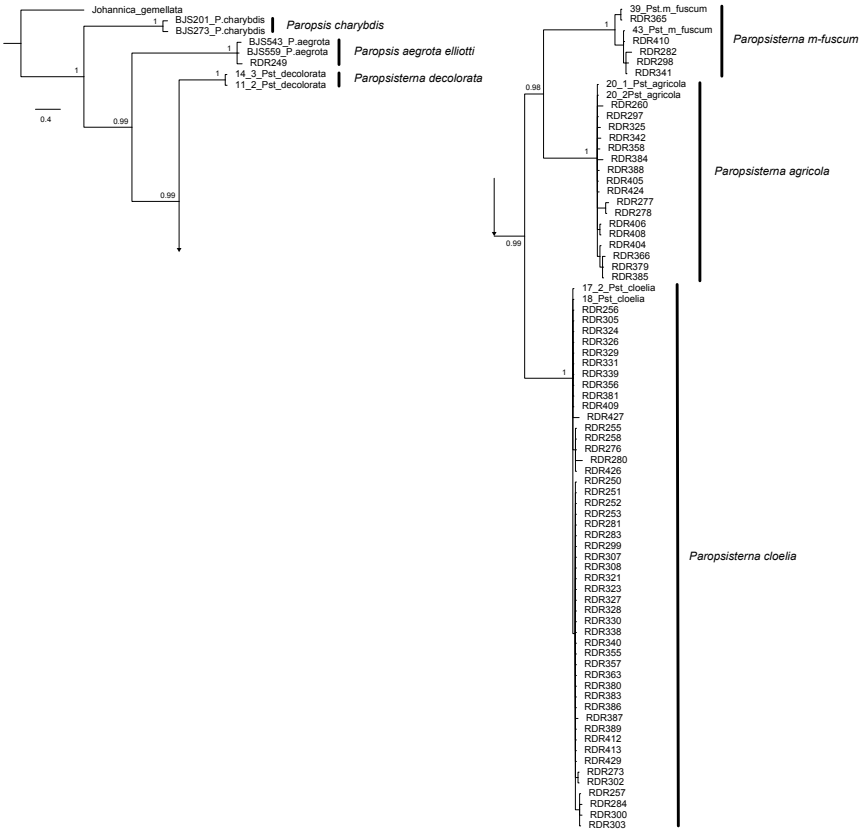
SI Table 2. List of all host beetle material examined along with their DNA voucher number, DNA voucher number of their associated parasitoid, and the unique identifier of the tree from which they were collected.

SI Table 3. List of all phytochemical compounds isolated, their identification, molecular weight, variable loading values for the first and second principal components, and the absolute value of the first subtracted

by the second principal component used to rank compounds in their contribution to the separation of *Eadya* observed in Figure 5D.







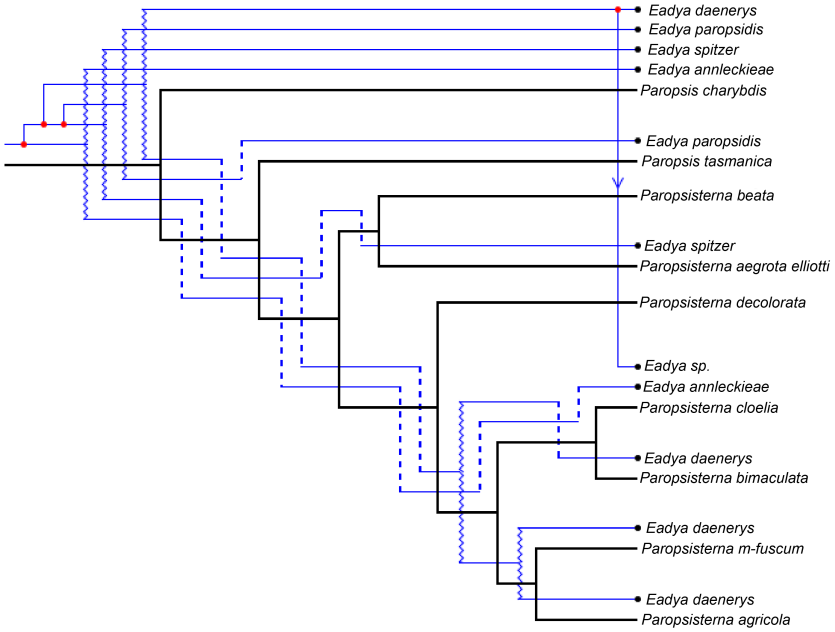


Table 1

	Eadya spitzer	*Eadya paropsidis*	*Eadya* sp. 1	*Eadya daenerys*	*Eadya annleckieae*
Eadya spitzer	0.0106				
Eadya paropsidis	0.0873	0			
Eadya sp. 1	0.0782	0.0857	0		
Eadya daenerys	0.1037	0.0986	0.0645	0.0014	
Eadya annleckieae	0.3042	0.3107	0.2809	0.2916	0.0027

Table 2

	Paropsis charybdis	*Paropsis aegrota elliotti*	*Paropsisterna decolorata*	*Paropsisterna m-fuscum*	*Paropsisterna agricola*	*Paropsisterna cloelia*
Paropsis charybdis	0.0076					
Paropsis aegrota elliotti	0.1826	0.0057				
Paropsisterna decolorata	0.1642	0.1528	0.0015			
Paropsisterna m-fuscum	0.1818	0.1735	0.1264	0.0078		
Paropsisterna agricola	0.1907	0.1508	0.1052	0.1136	0.0052	
Paropsisterna cloelia	0.1903	0.1605	0.1043	0.1132	0.0977	0.0020

Table 3

Eucalyptus globulus vs Eucalyptus sp. ANOVA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
PC1 ~ Species	1	333.3	333.3	24.09	3.56E-05	***
PC2 ~ Species	1	41.5	41.46	3.633	0.067	
PC1 Residuals	28	387.3	13.8			
PC2 Residuals	28	319.5	11.41			

Flush E. globulus Damaged vs Undamaged ANOVA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
PC1 ~ Damage Status	1	52.4	52.42	1.3	0.284
PC2 ~ Damage Status	1	30.47	30.47	1.513	0.25
PC1 Residuals	9	363	40.34		
PC2 Residuals	9	181.19	20.13		

Beetles ANOVA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
PC1 ~ Species	3	235.4	78.46	2.266	0.0862	
PC2 ~Species	3	381.6	127.19	26.1	3.09E-12	***
PC1 Residuals	90	3115.9	34.62			
PC2 Residuals	90	438.6	4.87			

Wasps ANOVA

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	
PC1 ~ Species	2	420.8	210.39	7.611	0.00139	**
PC2 ~Species	2	112.7	56.34	4.183	0.0214	*
PC1 Residuals	46	1271.6	27.64			
PC2 Residuals	46	619.6	13.47			

Table 4

Beetles PC2 Tukey

	diff	lwr	upr	p adj	
Pst. agricola ~ P. aegrota ellioti	1.0312873	-4.9061059	6.9686810	0.9685553	
Pst. cloelia ~ P. aegrota ellioti	5.3241401	-0.4954492	11.1437290	0.0853598	
Pst. m-fuscum ~ P. aegrota ellioti	-0.6345391	-7.0956958	5.8266180	0.9940020	
Pst. cloelia ~ Pst. agricola	4.2928528	2.7678001	5.8179050	0.0000000	***
Pst. m-fuscum ~ Pst. agricola	-1.6658264	-4.8603078	1.5286550	0.5244356	
Pst. m-fuscum ~ Pst. cloelia	-5.9586792	-8.9284759	-2.9888820	0.0000059	***

Wasps PC1 Tukey

	diff	lwr	upr	p adj	
E. daenerys ~ E. annleckieae	6.1833870	1.7967255	10.5700500	0.0037818	**
E. n. sp. ~ E. annleckieae	8.6180520	-0.6323013	17.8684000	0.0724585	
E. n. sp. ~ E. daenerys	2.4346650	-7.3533154	12.2226500	0.8195074	

Wasps PC2 Tukey

	diff	lwr	upr	p adj
E. daenerys ~ E. annleckieae	-2.843038	-5.90509	0.219015	0.073681
E. n. sp. ~ E. annleckieae	-5.486203	-11.94329	0.9708871	0.1101054
E. n. sp. ~ E. daenerys	-2.643166	-9.47554	4.1892086	0.6199953