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Community Structure and Undescribed Species Diversity in Non-Pollinating Fig Wasps Associated with the Strangler Fig *Ficus petiolaris*

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Abstract

Figs and their associated mutualistic and parasitic wasps have been a focus of intensive ecological and evolutionary research due to their diversity, unusual reproductive biology, and highly coevolved interspecific relationships. Due to the ecological dependence of their interactions, fig wasps were once considered to be fig-species specific and to cospeciate with their hosts, however, a growing body of evidence reveals mixed support for species specificity and the importance of additional evolutionary processes (e.g., host switching) structuring these long-term interactions. Our research on the genus *Idarnes* Walker, 1843 (Hymenoptera, Agaonidae), a common non-pollinating wasp of New World fig flowers, reveals a community in which multiple wasp species coexist on the same host in space and time. Using both molecular and morphological data, we identify five distinct *Idarnes* lineages associated with a single host fig species, *Ficus petiolaris* Kunth, 1817 (Rosales, Moraceae). A comprehensive phylogenetic analysis including *Idarnes* species from numerous host fig species reveals that the lineages associated with *F. petiolaris* do not form a monophyletic group but are distantly related, suggesting multiple independent colonization events and subsequent diversification. Morphological and ecological data provide support that the wasps are partitioning niches within the figs, explaining the coexistence of these diverse lineages on the same host fig. These results, coupled with a growing body of research on pollinating and non-pollinating fig wasps, bring into focus a more dynamic picture of fig and fig wasp coevolution and highlight how wasp lineage divergence and niche partitioning contributes to increased species diversity and community structure on a single fig host.

Key words: coevolution, *Idarnes*, non-pollinating fig wasp, host switching, *Ficus*

Coevolutionary interactions have had significant consequences for the diversity and organization of the Earth's biota (Futuyma and Slatkin 1983; Thompson 1982, 1994). Highly dependent relationships between two or more species may arise from their intimate interactions, sometimes leading to species-specific relationships. Histories of close interactions among species can result in congruent phylogenies if speciation in one partner (e.g., the host) results in speciation in the other partner (e.g., the parasite or pollinator; Page and Charleston 1998). There are numerous documented instances of symbiotic lineages in which phylogenetic histories appear to be significantly congruent (e.g., Hafner and Nadler 1988, Demastes and Hafner 1993, Moran and Baumann 1994, Hafner and Page 1995, McGeoch et al. 2000, Paterson et al. 2000, Thao et al. 2000, Clayton et al. 2003, Weiblen 2004,

Cruaud et al. 2012). Such parallel cladogenesis is indicative of a long history of species specificity, cospeciation, and correlated evolution. Obligate species interactions, however, do not necessarily produce congruent evolutionary histories. Evidence of incongruence between phylogenies reveals the broader set of evolutionary processes—including host switching and the speciation and extinction of individual symbiont taxa—that contribute to the diversification and biodiversity of symbiotic lineages (e.g., Satler et al. 2019). Processes that generate congruent (i.e., cospeciation) and incongruent (i.e., host switching) cophylogenetic patterns can contribute to the evolution of diverse associations in which hosts interact with one or more closely related symbionts, and the clade of symbionts comprises both host specialists and generalists (e.g., Stireman et al. 2012).

1

Plants and associated phytophagous insects are two of the most diverse groups of organisms, representing numerous, often highly specific, coevolutionary interactions (Futuyma and Agrawal 2009). Many of these interactions can be categorized into two general types: pollination mutualisms and plant–herbivore–parasite relationships. Particularly spectacular examples of both these relationships can be found between figs (family Moraceae, genus *Ficus*) and their associated wasps (Hymenoptera: Chalcidoidea). *Ficus* is one of the largest genera of land plants (Berg 1989, Cook and Segar 2010, Cruaud et al. 2012) with all 750+ described species producing urn-shaped infloresences lined internally with separate female and male flowers. These inflorescences, commonly called figs (technically syconia), are nurseries for the development of a suite of associated pollinating (mutualistic) and non-pollinating (antagonistic) wasps (Wiebes 1979, Compton and Hawkins 1992, Boucek 1993, Weiblen 2002, Cook and Rasplus 2003, Segar et al. 2013, Borges 2015). Fig plants are entirely dependent upon pollinating fig wasps (family Agaonidae) for pollination services, while these wasps are, in turn, entirely dependent on syconia for reproduction and larval development. In this mutualism, each fig species produces a distinct blend of floral volatile cues attracting a typically host-specific pollinator species to receptive syconia (Barker 1985, Hossaert-McKey et al. 1994, Ware and Compton 1994), often over extraordinary distances between low-density flowering hosts (Nason et al. 1996, 1998; Ahmed et al. 2009). These pollinator wasps enter syconia through a small terminal pore (ostiole), remove pollen from specialized morphological structures (pollen pockets; Galil and Eisikowitch 1969, Ramirez 1978, Wiebes 1979), pollinate, and oviposit through the style into a subset of female flowers, which are galled by developing larvae. Fig seeds and wasp offspring mature in synchrony weeks to months later, at which time wasps eclose and mate within syconia, with female wasps collecting pollen from now mature male flowers before dispersing in search of trees bearing new receptive syconia. This mutualistic interaction with pollinating fig wasps benefits both female and male fitness functions in the fig through pollen delivery and dispersal, respectively.

Given their highly dependent relationships, it was long assumed that figs and their pollinators were reciprocally species-specific with a history of cospeciation (Wiebes 1979, Weiblen 2002). These predictions were supported by morphological taxonomic work (Ramirez 1974, Wiebes 1979, Berg 1989) and molecular investigations (Machado et al. 1996, Lopez-Vaamonde et al. 2001, Machado et al. 2001, Weiblen 2001, Weiblen and Bush 2002, Ronsted et al. 2005, Jousselin et al. 2008, Moe et al. 2011, Cruaud et al. 2012) indicating support for the phylogenetic congruence of major fig and pollinator wasp lineages (i.e., sections of figs and genera of wasps). However, there is growing evidence that the phylogenetic congruence of fig and pollinator wasp lineages at deeper taxonomic levels is not reflected at shallower taxonomic levels (e.g., Molbo et al. 2003, Machado et al. 2005, Haine et al. 2006, Jackson et al. 2008, McLeish and Van Noort 2012, Darwell et al. 2014, Rodriguez et al. 2017, Yu et al. 2019), including a recent high-resolution study employing data from thousands of loci (Satler et al. 2019). Despite phylogenetic incongruence in evolutionary time, molecular data reveal a large majority of pollinator wasps to be highly host-specific in ecological time at a given geographic location. For example, in the vicinity of Barro Colorado Island in central Panama, 22 of 24 pollinator species are fig-species specific, with only two wasps associated with more than one host (Marussich and Machado 2007; E. A. Herre et al. unpublished data).

In contrast to the pollinators, non-pollinating fig wasps are more diverse (numerous families) and represent a broad suite of ecologies and life history strategies (Boucek 1993, Weiblen 2002, Cook and Rasplus 2003, Cruaud et al. 2011, Borges 2015, Duthie et al. 2015). Although less well studied than the pollinators, the community of non-pollinators associated with an individual fig species typically includes gallers of fig flowers, cleptoparasites, and parasitoids of fig pollinating or non-pollinating wasps (e.g., Weiblen 2002, Borges 2015, Farache et al. 2018). Many aspects of the life history and dispersal biology of these insects are similar to those of the pollinator, including attraction to fig floral cues and synchronized development with fig seeds. Unlike pollinators, however, most non-pollinators (including all Neotropical lineages) oviposit from the exterior of syconia, inserting their ovipositor through the syconium wall to exploit developing female flowers or wasps. Interactions with non-pollinating fig wasps incur fitness costs to figs and, often, to pollinators (Segar and Cook 2012).

Perhaps because their development has fewer constraints (not having to pollinate, or rely as heavily on chemical cues to find hosts, etc.; Cook and Segar 2010), non-pollinator wasps are more frequently oligophagous and associated with multiple fig species (as opposed to pollinator wasps), and these hosts more frequently support multiple congeneric non-pollinators (Weiblen and Bush 2002, Jousselin et al. 2006, McLeish et al. 2010, Krishnan and Borges 2014, Darwell and Cook 2017, Farache et al. 2018). For example, a mitochondrial barcoding survey of *Idarnes* (family Agaonidae, subfamily Sycophaginae) non-pollinators from 16 fig species in the Barro Colorado Island area conducted by Marussich and Machado (2007) found three *Idarnes* species in the *I. carme* species group (cleptoparasites) each associated with three fig host species, and two *Idarnes* species in the *I. flavicollis* species group (gallers of pistillate flowers) associated with two and four fig hosts, respectively. Similarly, they found two species of *Heterandrium* (likely cleptoprarasites or parasitoids; family Pteromalidae, subfamily Otitesellinae) each associated with three hosts, and three species of '*Aepocerus*' (large gallers; more recently understood to be *Ficicola*, family Pteromalidae, subfamily Otitesellinae; Farache et al. 2018) each associated with two hosts. These associations result in nine of the 16 fig species hosting two or more congeners of at least one of these wasp lineages. Farache et al. (2018) report even higher levels of generalism in a Brazilian non-pollinating fig wasp community, where 19 of 42 non-pollinator wasp morphospecies were recovered in more than one host fig species. While some of these fig and non-pollinator associations may reflect a long history of cospeciation, cases of oligophagy and multiple congeners associated with the same host likely represent outcomes of host switching. Together, these processes have resulted in up to 30 non-pollinator species associated with a single fig species (Compton and Hawkins 1992). Thus, there is growing emphasis on understanding the mechanisms enabling multiple closely related non-pollinators to coexist on the same fig host (Krishnan and Borges 2014; Duthie et al. 2014, 2015).

Here we investigate the community structure and mechanisms underlying the coexistence of five lineages of *Idarnes* associated with *Ficus petiolaris*, a New World strangler fig (subgenus *Urostigma*, section *Americana*; ca. 120 described species). We use mitochondrial DNA sequence data to determine phylogenetic relationships and sequence diversity among the sampled lineages of *Idarnes* wasps. Placed in a broader *Idarnes* phylogeny, we ask whether the sampled lineages diversified on their current host or if additional processes (i.e., host switching) have generated current *Idarnes* diversity associated with *F. petiolaris*. In addition, we use morphological and behavioral data to investigate how lineage diversity corresponds with phenotypic differences and how partitioning of host niche space may promote coexistence among *F. petiolaris* non-pollinating wasp species.

Materials and Methods

Host Species

The Sonoran Desert rock fig, *F. petiolaris*, is found in Sonoran Desert habitats of the Baja California peninsula and mainland Sonora, extending south through central Mexico and into Oaxaca. In contrast to the hemi-epiphytic or host-strangling habit of most *Urostigma* figs, this lithophytic species grows on rocky outcrops and cliff faces. Historically recognized as a complex of two to four species, including *F. brandegei*, *F. jaliscana*, *F. palmeri*, and *F. petiolaris* (Shreve and Wiggens 1964, Felger and Lowe 1970, Wiggens 1980, Carvajal et al. 2001), the Sonoran Desert rock fig is now recognized as a single species comprising two subspecies, *Ficus petiolaris palmeri* and *Ficus petiolaris petiolaris* (Felger et al. 2001, Piedra-Malagón et al. 2011). Subspecies *F. p. palmeri* is the only fig endemic to the Baja California peninsula but also occurs in coastal Sonora. In Sonora, subspecies *F. p. petiolaris* generally occurs away from the coast in more mesic habitats, in some locations with other strangler (*F. cotinifolia*, *F. crocata*, and *F. pertusa*) and free-standing (*F. insipida*, subgenus *Pharmacosycea*) fig species. *Ficus petiolaris* produces highly outcrossed and asynchronously developing fruit crops (Gates and Nason 2012) that support a diverse community of fig wasps, all taxonomically undescribed. We categorize these species based on morphology (i.e., morphospecies). These including a pollinator (*Pegoscapus* sp.) and five chalcidoid genera of non-pollinators, including four species of *Idarnes*, two species of *Heterandrium* (family Pteromalidae), one species of *Ficicola* (family Pteromalidae) and its parasitoid, a species of *Physothorax* (family Torymidae), and one species of *Sycophila* (family Eurytomidae).

Idarnes Species

Idarnes are the most abundant non-pollinating wasps attacking New World *Americana* figs (Gordh 1975, Hamilton 1979, Bronstein 1991, West et al. 1996, Farache et al. 2018) and comprise three species groups: the less speciose *I. incerta* group (gallers) and the more speciose *I. flavicollis* (gallers) and *I. carme* (cleptoparasites) groups (Gordh 1975, Boucek 1993, Cruaud et al. 2011, Farache et al. 2018). Despite their diversity and ecological dominance, as well as their negative impacts on pollinator production (West and Herre 1994), only ca. 15 of the *I. carme* and *I. flavicollis* species associated with the ca. 120 described *Americana* figs have been taxonomically described (Gordh 1975, Boucek 1993), none of which are associated with *F. petiolaris*. Based on morphology, *F. petiolaris* hosts one member of the *flavicollis* group and three members of the *carme* group. Three of these *Idarnes* (the *I. flavicollis* and two *I. carme*) co-occur within syconia throughout the range of this *F. petiolaris* in Baja California and Sonora, Mexico. We have labeled these three species as LO1 (*flavicollis*), SO1 (*carme*), and SO2 (*carme*), where LO and SO stand for Long Ovipositor and Short Ovipositor, respectively (Duthie et al. 2015, Duthie and Nason 2016). The fourth (an *I. carme* species labeled LO2) is normally associated with *Ficus pertusa* but has been found developing within *F. petiolaris* figs in Sonora where the two species co-occur.

In a survey of six Brazilian fig species, *Idarnes carme* species were found to arrive at syconia after the pollinator and most *I. flavicollis* wasps (Farache et al. 2018). Elias et al. (2012) found that an *I. carme* species associated with *F. citrifolia* in Brazil inserts its ovipositor through the walls of developing galls initiated by pollinator larvae. They conclude that *I. carme* larvae are cleptoparasites that kill pollinator lavae and feed on gall tissue. *Idarnes flavicollis* species arrive at syconia just prior to, during, or after the arrival the pollinator (Farache et al. 2018). Elias et al. (2008) also investigated

an *I. flavicollis* species associated with *F. citrifolia* that inserts its ovipositor through the syconium wall to oviposit through the style into pistillate flowers. They conclude *I. flavicollis* wasps to be gallers, as has been suggested previously based on more correlative evidence (West and Herre 1994). Females of both *Idarnes* species groups possess ovipositors three to six times their body length. The wingless males of these species groups fight for access to females using enlarged mandibles (Hamilton 1979, Murray 1989, West and Herre 1994), and fertilized females exit syconia—without collecting pollen—via holes made by males of the pollinator species.

Sampling

Idarnes wasps were collected from 16 *F. petiolaris* localities distributed across the Baja California peninsula and adjacent mainland Sonora (Fig. 1; Table 1). Thirteen of these host locales were *F. p. palmeri* (11 peninsular and 2 mainland) and three were *F. p. petiolaris* (mainland). Wasps were reared from mature syconia, or occasionally collected from the exterior of receptive female phase or mature male phase syconia, and stored in 95% ethanol. For molecular analysis, we sequenced a single wasp of each species group from each mature fruit. In total, 95 independent *Idarnes* individuals were analyzed for sequence variation in a portion of the mitochondrial cytochrome oxidase I (COI) gene.

DNA Extraction and Amplification

Genomic DNA was isolated from individual wasps using the Gentra Puregene Tissue Kit following the manufacturer's recommended protocol for single *Drosophila* extraction (Gentra Systems). Standard PCR amplification and sequencing reactions of the 3′ end of the mitochondrial gene COI were performed using the primers *Sw2618* (5′-GCTCATTTTCATTATGTTYTATCTATRGG-3′; Machado 1998) and *New Pat* (5′-TCCAATGCACTAATCTGC
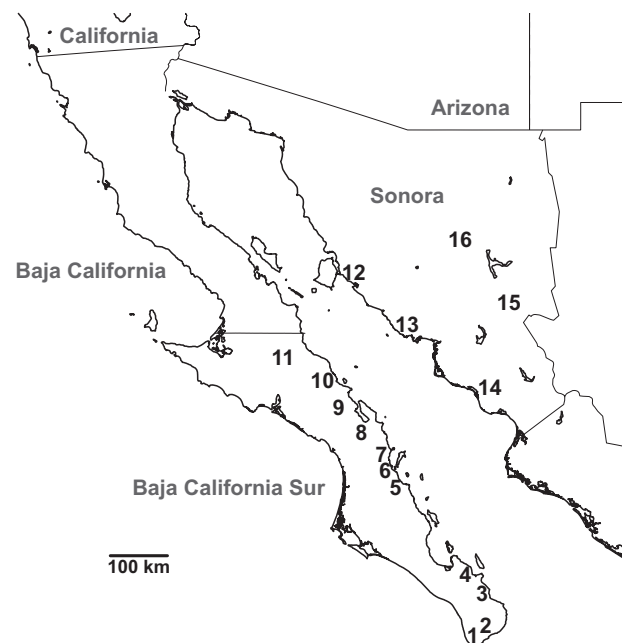

Fig. 1. Sampling sites for *Idarnes* wasps associated with *Ficus petiolaris*. *Idarnes* wasps were collected from host *F. petiolaris* localities distributed across Sonoran Desert habitats in Baja California (1–11) and Sonora (12–16), Mexico. Locales 1–13 are from *F. petiolaris* subspecies *palmeri* and locales 14–16 are from *F. petiolaris* subspecies *petiolaris*. See Table 1 for additional details.

Table 1. Locality information for *Idarnes* wasps collected from host *Ficus petiolaris*

Site	Site name	Host subsp.	Latitude / longitude	N
Baja California				
1	Cabo San Lucas	*F. p. palmeri*	22.92408 / –109.97872	3
2	San Jose del Cabo	*F. p. palmeri*	23.12982 / –109.74721	4
3	San Bartolo	*F. p. palmeri*	23.74212 / –109.82229	8
4	Fig Summit	*F. p. palmeri*	24.01794 / –110.09735	6
5	Agua Verde	*F. p. palmeri*	25.57437 / –111.17371	1
6	Loreto Beach	*F. p. palmeri*	25.84059 / –111.33179	3
7	Loreto Mountains	*F. p. palmeri*	25.97532 / –111.47649	5
8	Fig Canyon	*F. p. palmeri*	26.35778 / –111.80384	7
9	Mulege	*F. p. palmeri*	26.75220 / –112.17586	2
10	Santa Rosalia	*F. p. palmeri*	27.24018 / –112.37004	2
11	San Francisco	*F. p. palmeri*	27.57151 / –113.07616	9
Sonora				
12	Cerro Kino	*F. p. palmeri*	28.89978 / –112.00536	16
13	Nacapule	*F. p. palmeri*	28.01515 / –111.05335	8
14	Buaysiacoba	*F. p. petiolaris*	27.08546 / –109.67891	5
15	Tepoca	*F. p. petiolaris*	28.48525 / –109.35906	9
16	Rio Sonora	*F. p. petiolaris*	29.50435 / –110.16918	7

Eleven localities (50 wasps total) were from peninsular Baja California and five localities (45 wasps total) were from mainland Sonora, Mexico. All Baja and two coastal Sonoran locales represent *F. petiolaris* subspecies *palmeri*, while three inland Sonoran populations represent *F. petiolaris* subspecies *petiolaris*. *N* indicates the number of *Idarnes* sequenced per site.

CAT-3′; Marussich and Machado 2007). The PCR amplification procedure was as follows: 94°C for 2 min, 38 cycles of 94°C for 2 min, 50°C for 45 s, and 72°C for 1 min, and a final extension step of 72°C for 6 min. Big Dye terminator kits, version 3.1 (Perkin-Elmer), were used for the sequencing reactions of both forward and reverse strands. The sequencing temperature cycle was 96°C for 2 min, 45 cycles of 96°C for 30 s, 50–52°C for 45 s, and 60°C for 4 min. Sequenced samples were gel run on an ABI Prism 377 Automated Sequencer. Sequences were first aligned using AutoAssembler and further editing was completed in MacClade v.4.08 (Maddison and Maddison 2005).

Phylogenetic Analyses

BEAST v2.5.2 (Bouckaert et al. 2014) was used to estimate gene tree distributions for the *Idarnes* sequences sampled from *F. petiolaris*. Uncertainty in DNA substitution models was accounted for with bModelTest (Bouckaert and Drummond 2017). This approach uses reversible jump Markov chain Monte Carlo to marginalize over a set of substitution models while sampling model parameters from the posterior distribution. We included all substitution models that differentiated between transitions and transversions, resulting in 31 possible models. A relaxed clock lognormal model was used, with a birth-death model as tree prior. The chain was run for 100,000,000 steps, sampling every 10,000 steps, resulting in 10,000 samples from the posterior distribution. Log files were analyzed in Tracer v.1.7 (Rambaut et al. 2018) to check for convergence. Tree distributions were summarized as a maximum-clade credibility (MCC) tree.

To provide a broader phylogenetic perspective and test for monophyly of our sampled wasps, we downloaded all *Idarnes* COI mtDNA sequences from NCBI GenBank that contained data for the same part of the gene sampled from our ingroup individuals (Supp Table S1 [online only]). These *Idarnes* sequences are associated with 29 host fig species, including *F. petiolaris*. In addition, we sampled two *Idarnes* wasps reared from *Ficus pertusa* growing in sympatry with *F. petiolaris* at site 15. Based on morphology, these wasps belong to the *I. carme* group. For the complete *Idarnes* data set, all downloaded sequences were aligned with our newly generated

sequences in MAFFT v7.310 (Katoh and Standley 2013). The matrix was then edge trimmed to match our ingroup sequences, with any downloaded *Idarnes* samples not containing sequence information for that part of the COI gene removed. This resulted in a complete matrix of 357 sequences. The phylogenetic analysis was run in BEAST v2.5.2, as described above. For the complete *Idarnes* data set, the analysis was run for 1,000,000,000 steps, sampling every 100,000 steps, resulting in 10,000 samples from the posterior distribution. Log files were checked in Tracer v1.7 and the resulting gene tree distribution was summarized as an MCC tree.

Genetic Diversity and Demographic History

We used DendroPy v4.4.0 (Sukumaran and Holder 2010) to calculate several summary statistics of the *Idarnes* species associated with *F. petiolaris*, including nucleotide diversity (π), Watterson's theta (θ_w), and Tajima's *D*. Kimura 2-parameter corrected genetic distances for intraspecific and interspecific comparisons were calculated with PAUP* v4.0a166 (Swofford 2003). We also estimated Bayesian skyline plots for these species in BEAST v2.5.2. Skyline plots were used to compare effective population size dynamics through time among the *Idarnes* species. We used bModelTest as described above to account for uncertainty in the DNA substitution process. We placed a coalescent Bayesian skyline prior on the tree. Analyses were run for 50,000,000 steps, sampling every 5,000 steps, to generate a posterior distribution of 10,000 samples. Tracer v1.7 was used to test for convergence and estimate the Bayesian skyline plots. Since we do not have a good estimate of mutation rate for this gene from these species, we chose not to calibrate the analyses to correlate effective population size changes with real time. Assuming similar mutation rates and generation times, we are able to compare relative population size dynamics through time among the species. Of note, we did not generate summary statistics or Bayesian skyline plots for LO2. The comprehensive *Idarnes* phylogeny revealed LO2 to comprise two cryptic species (see Results), rendering sample sizes too small for either of these cryptic species. And because we did not know of the two cryptic lineages in LO2 until after genetic study, they were not distinguished in the following morphological and behavioral analyses.
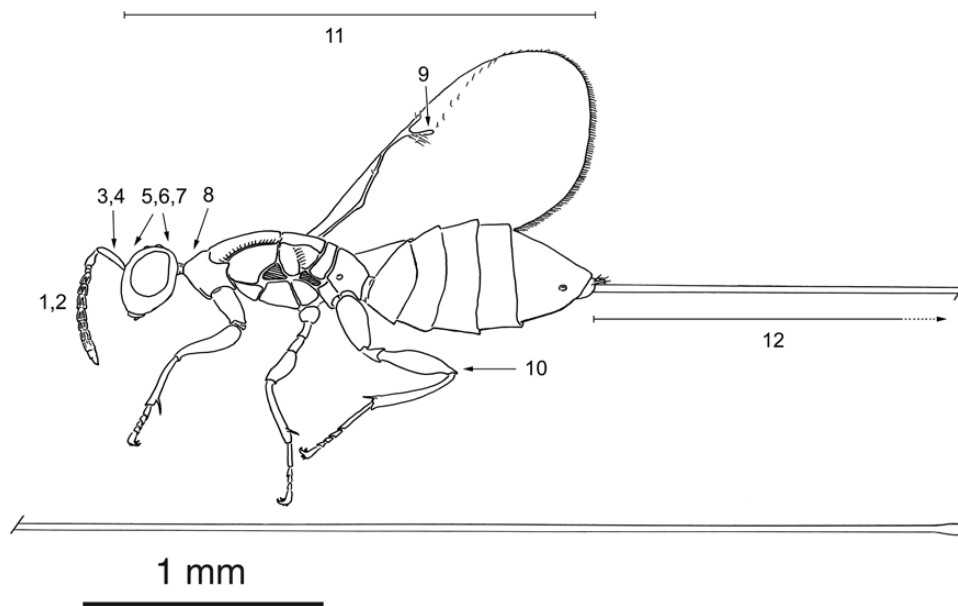
Fig. 2. A habitus drawing of an *Idarnes* wasp. The drawing represents a wasp from the LO1 species associated with host *Ficus petiolaris*. Illustrated are the 12 morphological characters measured for analysis. The characters are as follows: 1) presence of antennal setulae, 2) number of antennal segments, 3) scape length, 4) scape color (amber vs dark), 5) head width, 6) inter-antennal distance, 7) facial width, 8) collar length, 9) stigmal vein length, 10) femur color (amber vs dark), 11) body length, and 12) ovipositor length.

Morphological and Behavioral Characterizations

To statistically quantify morphological variation and to test associations between phenotype and species, we conducted multivariate morphological analysis on a geographically and morphologically diverse representation of individuals. Our analysis was based on the following 12 morphological characters: 1) presence of antennal setulae, 2) number of antennal segments, 3) scape length, 4) scape color (amber vs dark), 5) head width, 6) inter-antennal distance, 7) facial width, 8) collar length, 9) stigmal vein length, 10) femur color (amber vs dark), 11) body length, and 12) ovipositor length (Fig. 2). Characters 3, 5–9, 11, and 12 were continuous, while characters 1, 2, 4, and 10 were ordinal. In total, measurements were obtained for 11 (LO1), 6 (LO2), 11 (SO1), and 11 (SO2) independent wasps. Multivariate differences in morphology were visualized using principle components analysis (PCA). We used a Mantel test to determine the significance of correlations between morphology (Euclidean distance matrix) and species identity (design matrix). Analyses were conducted using the program NTSYSpc ver. 2.1 (Exeter Software).

We investigated two dimensions of potential behavioral differentiation among *Idarnes* lineages. To determine whether LO1, LO2, SO1, and SO2 wasps oviposit into syconia at different developmental stages, we identified ovipositing wasps and collected the syconia they were ovipositing into to determine its developmental stage. Based on the condition of stigmas and ovules, syconia developmental stages were categorized as early female phase (female flowers just reaching anthesis), mid female phase (female flowers in full anthesis), late female phase (stigmas of female flowers beginning to senesce), and interphase (stigmas senesced, ovaries expanding). For reference, the pollinator arrives primarily at mid female phase. The timing of oviposition was assayed for LO1, SO1, and SO2 wasps at Site 4, and for LO2 at Site 13 (Fig. 1; Table 1). We used a Chi-squared test to see if wasp species differed in the state of syconia in which they oviposited.

In a second analysis conducted using samples collected at Site 8 in Baja California (where LO2 wasps are absent), we determined whether *I. carme* and *I. flavicollis* wasps developed within different ranks of seeds within *F. petiolaris* syconia. We collected and preserved (in ethanol) late-developmental stage syconia prior to wasp eclosion. From these, we removed galls containing developing wasps, organizing them into three ranks: inner-most, intermediate, and outer-most. Wasps were dissected from each rank and identified at the level of *I. flavicollis* wasps (LO1) and *I. carme* wasps. We treated *I. carme* wasps as the same (SO1 plus SO2) since these two morphospecies could not be consistently distinguished at this stage of development and are presumed to be filling the same role ecologically. We used a Chi-squared test to see if wasp species differed in the location they developed within syconia.

Results

DNA Sequence Data

Ninety-five sequences were generated from the *Idarnes* wasps associated with *F. petiolaris*. This resulted in a DNA matrix of 323 base pairs. All newly developed sequence data have been deposited at GenBank under accession numbers MN863389–MN863485.

Phylogenetic Analyses

Phylogenetic analysis recovers four strongly supported clades (Fig. 3). Three of these clades (SO1, SO2, and LO2) are associated with the *I. carme* species group and one (LO1) with the *I. flavicollis* species group. The LO1 clade is further separated into largely distinct Baja and Sonora groupings. Varying levels of support are seen throughout the tree, but essentially no support is recovered for the interrelationships among the LO1, LO2, SO1, and SO2 clades. Placed in a broader phylogenetic context, these four clades associated with *F. petiolaris* do not form a monophyletic group, but are distributed throughout the *Idarnes* phylogeny (Fig. 4). The LO1, SO1, and SO2 groups are monophyletic with strong support (pp = 1.0). In contrast, the LO2 group is not monophyletic in the larger phylogeny, but rather forms two clades (both pp = 1.0) with other host-associated wasps interspersed between them (Fig. 4). These LO2 lineages were sampled from both *F. petiolaris* and *F. pertusa*, but are only found
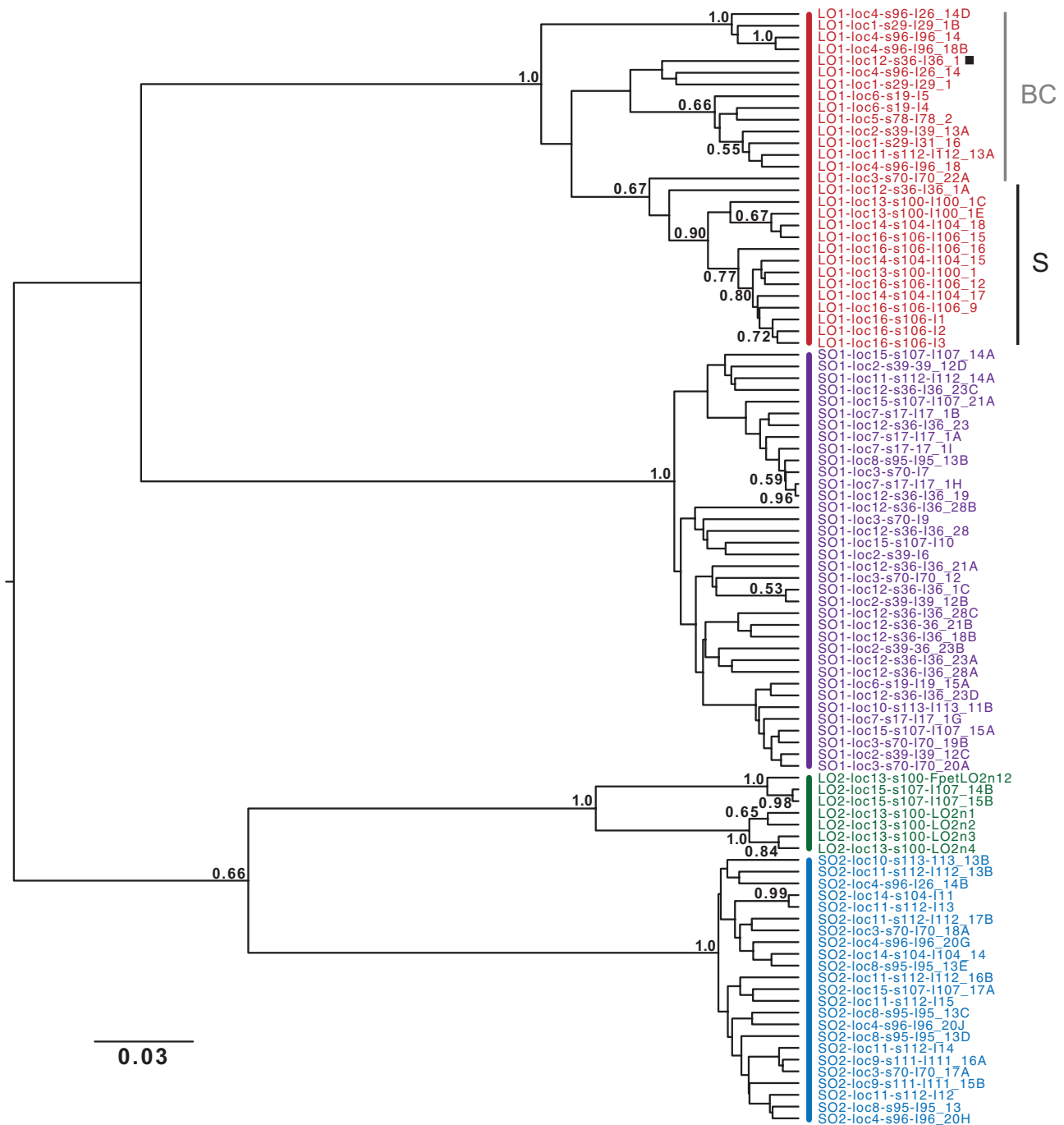
Fig. 3. Maximum-clade credibility tree for *Idarnes* mtDNA sequences from wasps associated with *Ficus petiolaris*. Four distinct clades (LO1, SO1, LO2, SO2) each with a posterior probability of 1.0 were recovered. There is little information in the data as to how these four clades are related. Posterior probabilities ≥0.50 are presented. Taxon names are composed of clade name, locality number, and internal lab numbers. In addition, LO1 sequences show phylogeographic structure, with sequences primarily clustering based on if sampled from Baja California (BC) or Sonora (S). One sequence (denoted with black box) is an exception, where the wasp was sampled from Sonora (locale 12; see Fig. 1) yet clusters with Baja California sequences.

on the former where the two fig species co-occur. More generally, the broader *Idarnes* phylogeny demonstrates that wasps within the *F. petiolaris* system do not form a monophyletic group but represent distant and distinct evolutionary lineages.

Genetic Diversity and Demographic History

LO1 possesses substantially higher sequence diversity than SO1 or SO2, both in terms of number of segregating sites (76 out of 323), nucleotide diversity ($\pi = 0.0515$), and Watterson's theta ($\theta_w = 0.0599$)

(Table 2). In addition, LO1 contains much higher average intraspecific genetic divergence (5.48%) than either SO1 (1.14%) or SO2 (0.48%), including a maximum intraspecific pairwise comparison of 18.42% reflecting phylogeographic structure within the clade. There is an expected linear relationship between these measures of genetic diversity and effective population size (N_e) so that, given similar mutation rates, N_e for LO1 is substantially higher than for the two other species. All three tested groups (LO1, SO1, SO2) had negative Tajima's *D* values. In addition, the higher N_e in LO1 was reflected in the Bayesian skyline
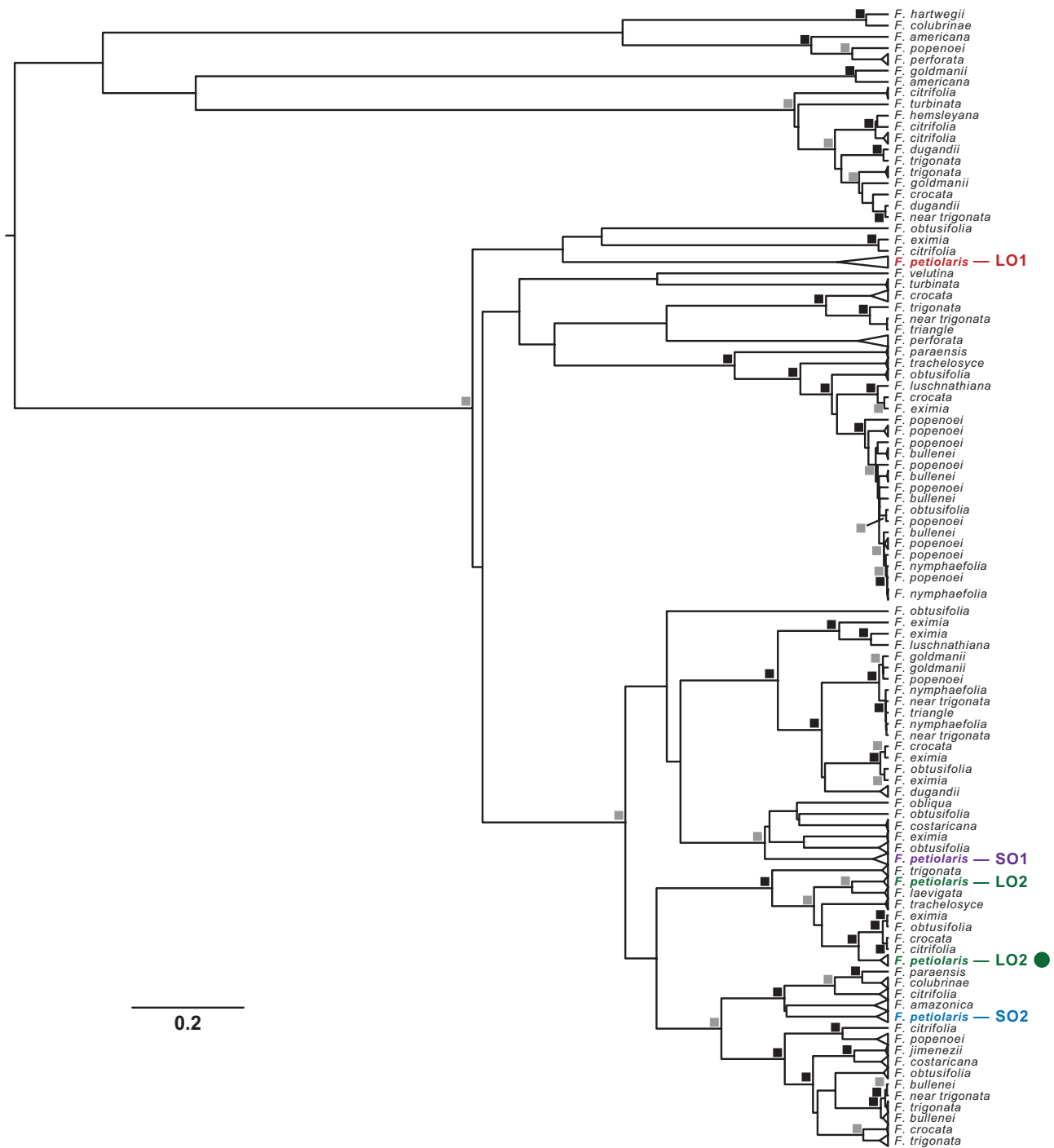
Fig. 4. Maximum-clade credibility tree representing the complete *Idarnes* data set. Tips of the tree are labeled with the host fig taxon name. The four *Idarnes* species associated with *Ficus petiolaris* do not form a monophyletic group, suggesting a history of host switching for the *Idarnes* species associated with *F. petiolaris*. Additionally, LO2 is not monophyletic but forms two distinct clades, with *Idarnes* sampled from other host fig trees interspersed between them. The two *Idarnes* samples collected from *Ficus pertusa* in sympatry with *F. petiolaris* cluster with one of the LO2 clades (denoted by a circle). Black squares represent posterior probabilities ≥0.95; gray squares represent posterior probabilities <0.95 and ≥0.5. All other nodes had support values below 0.5. Wasps that formed monophyletic groups and were associated with the same host fig species were collapsed for visual purposes.

plots (Fig. 5). SO1 and SO2 were characterized by constant population size with slight increases through time. LO1, however, was starkly different, reflecting a recent and rapid population size expansion.

Morphological and Behavioral Analyses

Gross anatomical surveys of the *Idarnes* specimens revealed four distinct morphotypes corresponding to LO1, LO2, SO1, and SO2.

The most visually obvious distinguishing feature of these groups is ovipositor length: two of the *I. carme* morphotypes (SO1 and SO2) possess relatively short ovipositors compared to the third *I. carme* morphotype (LO2) and the *I. flavicollis* morphotype (LO1) (Table 3). The distribution of ovipositor length for these two pairs of species (SO1 and SO2 vs LO1 and LO2) was non-overlapping.

Table 2. Summary statistics for three clades (LO1, SO1, SO2)

Clade	N	SS	π	θ_w	Tajima's D	Intraspecific (%)	Interspecific (%)
LO1	29	76	0.0515	0.0599	−0.7001	5.48 (0–18.42)	20.71 (15.93–30.23)
SO1	36	22	0.0111	0.0164	−1.1693	1.14 (0–4.59)	17.95 (13.18–29.96)
SO2	23	11	0.0048	0.0092	−1.6658	0.48 (0–2.60)	17.82 (13.18–30.23)

LO2 was not included since it contains two cryptic species and samples sizes are too small if each species were analyzed independently. Values include number of sequenced individuals (*N*), number of segregating sites (SS), nucleotide diversity (π), Watterson's theta (θ_w), Tajima's *D*, and average (minimum–maximum) Kimura two-parameter corrected genetic distance for both intraspecific and interspecific comparisons.
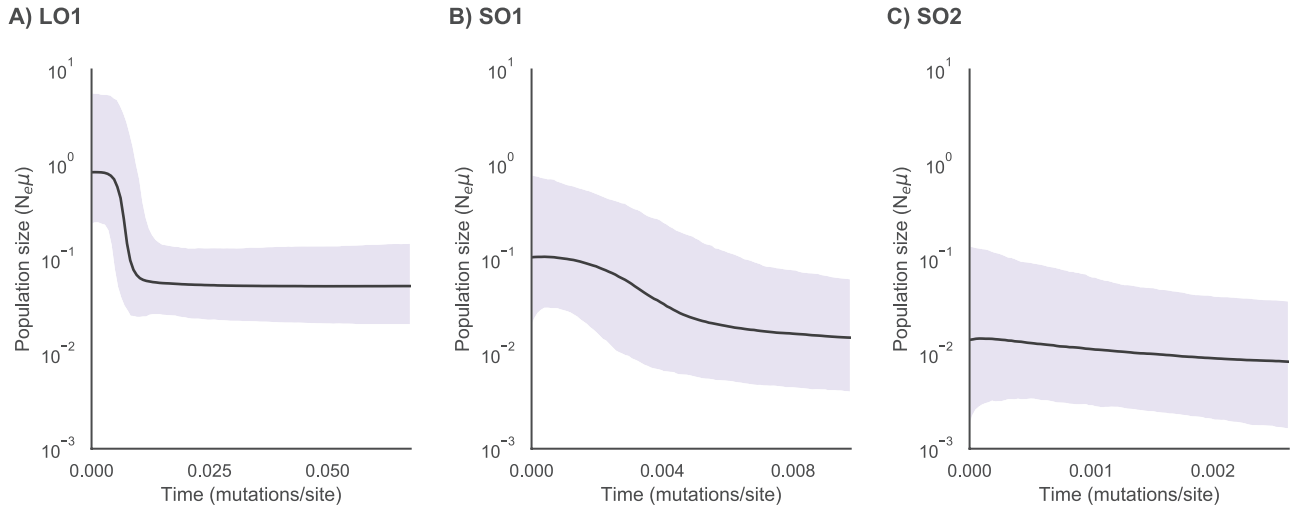

Fig. 5. Bayesian skyline plots for three *Idarnes* species. *X*-axes are in units of mutations per site, while *y*-axes are in units of effective population size scaled by mutation rate. LO1 shows sharp growth in population size, whereas SO1 and SO2 show a similar pattern of consistent population size through time with minimal growth. LO2 was not included as it contains two cryptic species reducing sample sizes too low for analysis.

Table 3. For each of the four *Idarnes* mtDNA clades (LO1, LO2, SO1, and SO2), means and standard errors (for continuous variables) of measurements of each of 12 morphological characters

	Idarnes morphotype					PCA Loadings	
Characters	LO1 (Baja) (*n* = 5)	LO1 (Sonora) (*n* = 6)	LO2 (*n* = 6)	SO1 (*n* = 11)	SO2 (*n* = 11)	PC1	PC2
1. Antennal setulae	1.8	1.4	3	1.2	2.9	−0.126	−1.21
2. Anten. segments	13	13	13	12	12	0.800	−4.269
3. Scape length	0.18 (0.01)	0.175 (0.018)	0.127 (0.009)	0.119 (0.007)	0.151 (0.013)	0.781	0.456
4. Scape color	2.2	1.2	4	4	2.1	−0.561	−0.616
5. Head width	0.513 (0.041)	0.49 (0.042)	0.44 (0.021)	0.45 (0.026)	0.43 (0.014)	0.764	0.175
6. Inter-anten. dist.	0.097 (0.009)	0.094 (0.01)	0.11 (0.008)	0.103 (0.01)	0.099 (0.009)	−0.195	−0.512
7. Facial width	0.031 (0.002)	0.04 (0.006)	0.073 (0.006)	0.066 (0.009)	0.075 (0.013)	−0.820	−0.597
8. Collar length	0.129 (0.015)	0.101 (0.009)	0.076 (0.009)	0.071 (0.006)	0.085 (0.008)	0.846	0.305
9. Stigmal vein length	0.128 (0.025)	0.118 (0.006)	0.12 (0.01)	0.14 (0.015)	0.16 (0.016)	−0.424	0.547
10. Femur color	3	1.8	3	1	1	0.698	−6.16
11. Body length	2.01 (0.12)	1.83 (0.11)	1.77 (0.15)	1.78 (0.09)	1.74 (0.13)	0.617	0.427
12. Ovipos. length[a]	5.2 (0.36)	4.63 (0.29)	4.52 (0.34)	2.87 (0.20)	2.68 (0.21)	0.902	−0.351

LO1 is further split into Baja and Sonora to reflect the morphological divergence within the species.

[a]Ovipositor length sample sizes are 17, 6, 8, 17, and 17, respectively.

A PCA of 12 morphological characters further distinguished among the *Idarnes* morphotypes. The first and second principal axes explained 50.2 and 28.9% of the morphometric variation, respectively, and visually separated the four morphotypes into distinct clusters (Fig. 6). The PCA further separated LO1 into distinct Baja and Sonora groupings, largely consistent with mtDNA divergence (Fig. 3). A Mantel test revealed the correlation between phenotype and species to be highly significant (*P* < 0.0001). Morphological characteristics with the highest loadings in the PCA are indicated in Table 3.

Idarnes species displayed a significant difference in the developmental phase of syconia in which they oviposited (χ^2 = 89.859; df = 9; *P* < 0.001). SO1, SO2, and LO2 wasps (*carme* species) were primarily observed ovipositing in interphase fruits, although there were SO2 wasps observed ovipositing in early, mid, and late female phase fruits (Fig. 7A). In contrast, LO1 wasps (*flavicollis*) were primarily ovipositing in female phase fruits (early, mid, and late) with less than 25% ovipositing in interphase fruits. These wasps also showed significant differences in oviposition location within fruits (χ^2 = 9.823; df = 2; *P* = 0.007). Almost half of all LO1 wasps were
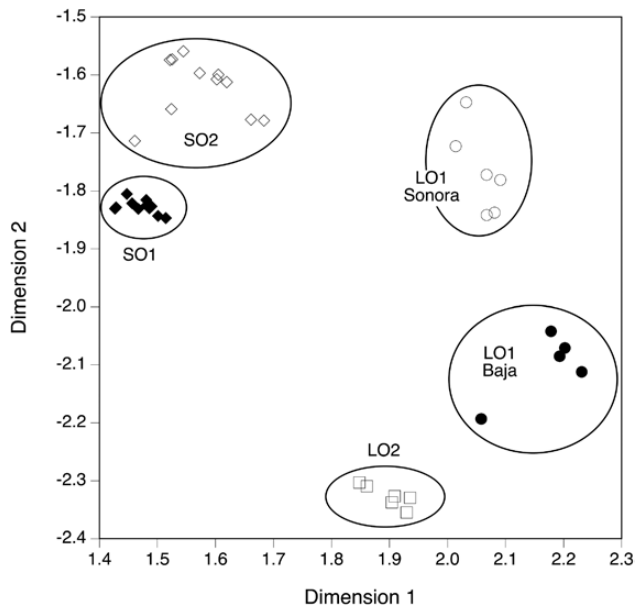
Fig. 6. Phenotypic variation among the four species represented by the first two coordinate axes of a principal component analysis. Twelve morphological characters were analyzed (Table 3). A Mantel test of the multivariate morphological differences among species was highly significant ($P < 0.0001$). In addition, LO1 shows further differentiation based on geography, with distinct clusters recovered for both Baja and Sonora samples.

recovered from the inner-most rank of galls within the syconia, as opposed to only 25% of SO1 and SO2 wasps (Fig. 7B). In contrast, a higher proportion of SO1 and SO2 wasps were collected from the middle and outer ranks of galls than LO1 wasps.

Discussion

Here we explored species diversity and interrelationships in the non-pollinating *Idarnes* wasps associated with *F. petiolaris*. Molecular and morphological data demonstrate the presence of five species of *Idarnes* associated with *F. petiolaris* in the Sonoran Desert of Baja California and Sonora, Mexico. Placed in a broader phylogenetic context, these species are distantly related, suggesting the importance of host shifting as a process for generating non-pollinator wasp diversity in this community. This study highlights the complexity of community structure and undescribed species diversity among non-pollinating fig wasps, how divergence along multiple axes (molecules, morphology, ecology) promotes coexistence on a single host fig species, and suggests that much concerning the origin and maintenance of the wasp communities remains to be understood.

Initially, morphological data suggested the presence of four species of *Idarnes* associated with *F. petiolaris*. Molecular data supported the four species hypothesis, as each morphospecies was monophyletic with strong support (pp = 1.0) in the gene tree (Fig. 3). Importantly, however, was how our interpretations changed once we included *Idarnes* species sampled from additional fig hosts (Fig. 4). The comprehensive phylogeny not only revealed our four *Idarnes* species to
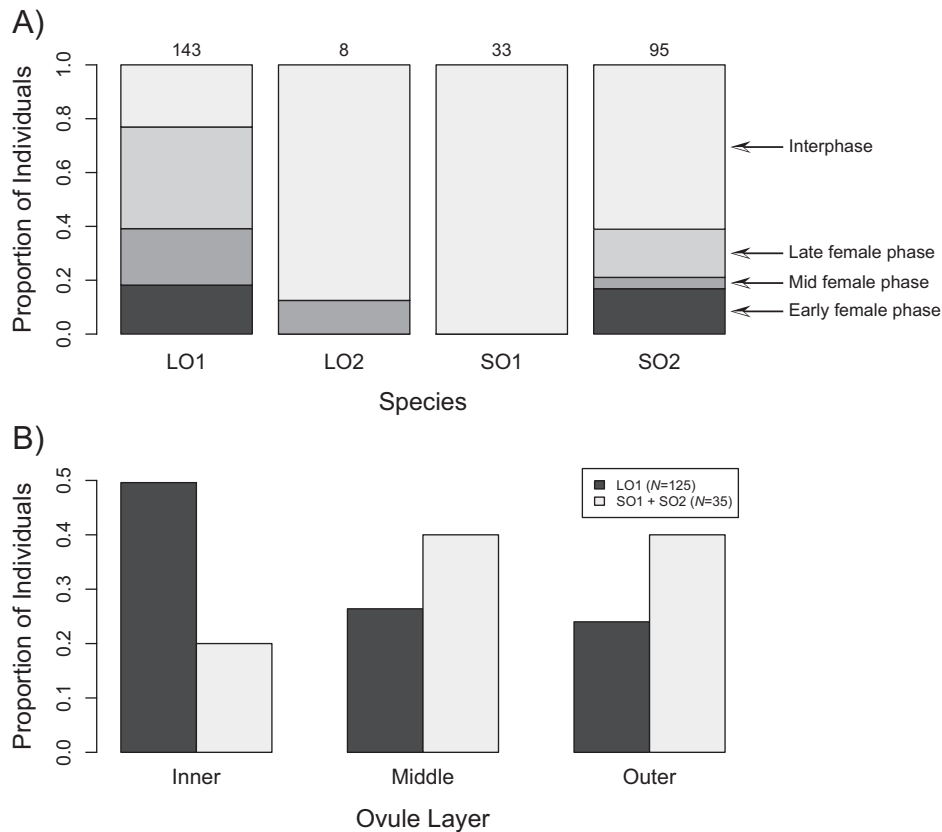

Fig. 7. *Idarnes* behavioral assays. (A) The proportion of the total number of *Idarnes* individuals of each species (*N* at top) observed ovipositing into syconia of each of four developmental stages, from early female phase to interphase (labeled on right). (B) The proportion of *I. flavicollis* (LO1) and *I. carme* (SO1+SO2) wasps developing in inner, middle, and outer ranks of ovules within syconia.

be distantly related but also that the LO2 morphospecies is composed of two cryptic species. The phylogenetic distance between these two species strongly supports them as distinct, and raises questions regarding the processes generating their similarity in morphometric space. Although the single gene sampled here is insufficient for resolving deeper phylogenetic relationships among *Idarnes*, it strongly supports three of our morphospecies as genetically distinct species (LO1, SO1, SO2) and uncovered a pair of cryptic species in what was initially identified as a single species based on morphology (LO2).

The existence of multiple *Idarnes* species groups attacking a single host has previously been documented (Marussich and Machado 2007, Farache et al. 2018). For example, West and Herre (1994) and West et al. (1996) sampled from six Panamanian strangler fig species, finding two cases in which *Idarnes* of the *I. carme* or *I. flavicollis* species occurred with members of the *I. incerta* species group on the same host fig species. This latter species group is different morphologically from the *I. carme* and *I. flavicollis* groups, being characterized by winged males and females with much shorter ovipositors (<0.5 mm; Boucek 1993). The *I. incerta* group is not found to be associated with *F. petiolaris*. Rather, *Idarnes* sampled from *F. petiolaris* represent *flavicollis* and *carme* species groups, species that differ more subtly in ovipositor length and other morphological characters (Fig. 6; Table 3). Differences in morphology and niche space likely promote coexistence in these sympatric *Idarnes* lineages, as has been documented in other non-pollinating fig wasp communities (e.g., Darwell and Cook 2017). While we here describe *Idarnes* diversity in the northern third of the range of *F. petiolaris*, additional *Idarnes* species may likely occur elsewhere in the broader distribution of this host in Mexico.

The possibility of multiple *Idarnes* species on one host fig was first raised by Hamilton (1979) in his studies of life-history traits in fighting male *Idarnes* wasps. Hamilton noted distinct female morphotypes with differences in ovipositor lengths associated with a Brazilian fig, but refrained from calling each type a different species because of the continuous variation found within the male wasps. Since then, only a few studies have combined morphological and molecular data to describe multiple *Idarnes* species groups associated with individual fig species, often with an *Idarnes* species group represented by two or more species (Marussich and Machado 2007, Farache et al. 2018). Combined with the evidence we present, these studies document 27 *I. carme*, 10 *I. flavicollis*, and 7 *I. incerta* species associated with 23 *Ficus* species. These studies reveal the coexistence of multiple lineages of *Idarnes* on individual fig species to be more common than previously recognized. Future studies sampling *Idarnes* communities associated with additional *Ficus* species will likely reveal further undescribed wasp species diversity.

Unlike pollinators, non-pollinating fig wasps are not constrained to function as pollen vectors for the host fig. Such constraints on pollinators should serve to strengthen coevolutionary relationships between these insects and their plant hosts, making extreme specialization and host tracking much more likely. With the finding that strict cospeciation has been relaxed between figs and their mutualistic partners (e.g., Molbo et al. 2003, Marussich and Machado 2007, Jackson et al. 2008, Cook and Segar 2010, McLeish and Van Noort 2012, Satler et al. 2019), it is not surprising to find similar relaxed relationships between figs and their wasp parasites. The hypothesis that non-pollinators are more likely to deviate from a pattern of strict cospeciation than are pollinators was supported by Weiblen and Bush (2002). In a study of Old World figs, *Sycomorus* sensu lato, and its mutualistic and parasitic agaonid wasps, Weiblen and Bush (2002) found significant evidence of cospeciation between *Ficus* and their pollinator lineages (*Ceratosolen*), but not between

Ficus and a floral parasite (*Apocryptophagus*). Additional studies have also demonstrated that it is not uncommon for non-pollinator fig wasps to attack two or more hosts (Marussich and Machado 2007, McLeish et al. 2012, Segar et al. 2012, Zhou et al. 2012, Farache et al. 2018). That these examples exhibit complex evolutionary dynamics, including cryptic within-host speciation and host shifting, suggests that other classic examples of host–parasite relationships and mutualisms may also exhibit a degree of evolutionary complexity that challenges our notions of highly coevolved interactions leading to cospeciation.

Given multiple *Idarnes* associated with a single host fig species, how do distinct species utilizing the same resources (fig fruit) coexist ecologically? One possible ecological mechanism for coexistence is that these species utilize the fig at different stages in maturity. For example, wasps with short ovipositors have an advantage ovipositing in syconia early in development when the fruit wall is thinner, while wasps with long ovipositors are the only type able to access female flowers later in development when the fruit wall is thicker (Kerdelhué et al. 2000, Weiblen and Bush 2002, Cruaud et al. 2011). Although we found significant differences in ovipositor length (Table 3) and in the developmental stage of syconia that *Idarnes* species were observed on in the field (Fig. 7A), we did not find support for the hypothesis that *Idarnes* with shorter ovipositors use younger syconia for oviposition. To the contrary, if we compare wasps arriving to early female phase and mid female phase fruits versus wasps arriving to late female phase and interphase fruits, SO1 and SO2 *Idarnes* with short ovipositors used significantly older syconia than LO1 and LO2 *Idarnes* (χ^2 = 89.859; df = 1; *P* < 0.001). This pattern was also observed by Elias et al. (2008), where *Idarnes* of the *carme* group (shorter ovipositors) consistently arrived to syconia later in development than *Idarnes* of the *flavicollis* group (longer ovipositors). Elias et al. (2012) suggest ecology explains the temporal partitioning, where the early arrivers are gallers while the *I. carme* species group are cleptoparasites, not creating new galls but rather taking advantage of already developing galls. Such an explanation is consistent with our observation of the placement of developing wasps within syconia, where *I. flavicollis* wasps (LO1) largely develop in the innermost rank of flowers while *I. carme* wasps (SO1 and SO2) largely develop in the middle and outer ranks (Fig. 7B). This suggests that LO1 wasps utilize similar resources as the pollinators and may be more directly competing with them, while SO1 and SO2 wasps attack already developing galls closer to the fig wall.

A contributing explanation for the coexistence of *Idarnes* species stems from the strong metapopulation dynamics they experience, with frequent extinction and recolonization of trees or local populations of trees. Tradeoffs between colonization and competitive abilities may permit species coexistence under this scenario of frequent population turnover (Tilman 1994). In a theoretical study, Duthie et al. (2014) showed how, when the dispersal distance to resource patches (such as receptive fig trees) is fluctuating, a dispersal ability and fecundity trade-off can facilitate coexistence of competing species (such as fig wasps) in ephemeral patch communities. In *F. petiolaris*, two lines of evidence are consistent with this prediction. First, though LO1 wasps are most abundant across sample locations, there is large variance across trees and populations in the abundance of each *Idarnes* morphotype reared from mature syconia (Piatscheck and Nason, unpublished data). Second, Duthie et al. (2015) have found an apparent trade-off between fecundity and dispersal ability, with LO1 wasps having a 50% greater egg load than SO1 and SO2 wasps, while these latter species have lower wing loading and a higher local colonization index indicative of greater capacities for dispersal. At a larger geographic scale, LO1 wasps show strong

phylogeographic differentiation between mainland and Baja peninsula samples that is absent in the other *Idarnes* species ([Fig. 3](#)). And related to their greater abundance and egg load (an indicator of competitive ability), LO1 wasps exhibit substantially larger effective population size ([Fig. 5](#)) and genetic diversity ([Table 2](#)) than do the other *Idarnes* species. Clearly, the coexistence of *Idarnes* species on an individual fig host is supported through multiple, simultaneously acting mechanisms, including trait and ecological niche divergence ([Darwell and Cook 2017](#)). Key evidence typically comes from the study of a single fig host community or an individual *Idarnes* species, however, and to generalize these mechanisms and determine their relative importance, future studies are needed of *Idarnes* in broader geographical contexts and across a greater diversity of fig host species.

Conclusions

Five species of non-pollinating wasps from the genus *Idarnes* are associated with the Sonoran Desert rock fig, *F. petiolaris*. These species are not closely related, suggesting host switching and subsequent diversification has played an important role in generating the *Idarnes* community associated with *F. petiolaris*. In addition, two of these species are morphologically cryptic and were only differentiated from each other with the use of molecular data, notably, from *Idarnes* associated with additional host fig species. Morphological and ecological differences allow the *Idarnes* community to coexist on the same host species while competing for limited resources (female flowers or galled female flowers). These results present a complicated picture of evolutionary diversification and species coexistence of non-pollinating fig wasps and highlight the role of niche partitioning in promoting community structure and diversity on a single host fig.

Supplementary Data

Supplementary data are available at *Insect Systematics and Diversity* online.

Acknowledgments

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