

DNA Barcoding Reveals Generalization and Host Overlap in Hummingbird Flower Mites: Implications for the Mating Rendezvous Hypothesis

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ABSTRACT: Hummingbird flower mites are assumed to monopolize single host plant species owing to sexual selection for unique mating rendezvous sites. We tested the main assumption of the mating rendezvous hypothesis—extreme host specialization—by reconstructing interactions among tropical hummingbird flower mites and their host plants using DNA barcoding and taxonomic identifications. We collected 10,654 mites from 489 flowers. We extracted DNA from 1,928 mite specimens and amplified the cytochrome c oxidase I (COI) DNA barcode. We analyzed the network structure to assess the degree of generalization or specialization of mites to their host plants. We recorded 18 species of hummingbird flower mites from three genera (*Proctolaelaps*, *Rhinoseius*, and *Tropicoseius*) interacting with 14 species of plants. We found that generalist mites are common, and congeneric mite species often share host plants. Our results challenge the assumption of strict specialization that supports this system as an example of mating rendezvous evolution.

Keywords: Costa Rica, hummingbird flower mites, La Selva Biological Station, reproductive isolation, tropical rain forest, plant-animal interactions.

Introduction

Interactions between hummingbirds and flower mites are a classic example of the diversity of adaptations and the complexity of life histories in the tropics. Hummingbird flower mites (family Ascidae, genera *Proctolaelaps*, *Rhinoseius*, and *Tropicoseius*; De Moraes et al. 2016) are nectar robbers that exploit mutualistic associations between hummingbirds

and plants. Hummingbird flower mites feed and mate inside flowers visited by hummingbirds (Colwell 1973). Mites can disperse by walking to flowers in the same inflorescence (Dobkin 1984), but to disperse among inflorescences or across host plants, mites require hummingbirds for transportation (Colwell 1973). When a hummingbird visits a plant, mites will climb its beak and into the nares, where they remain until arriving at their next host plant (Colwell et al. 1974; fig. 1).

Previous studies based on morphology reported that flower mites are highly specialized and hummingbird-pollinated flowers are usually colonized by a single mite species (Colwell 1986a, 1986b). In the few cases when more than one mite species share the same host plant, mite species are expected to belong to different genera to avoid hybridization with closely related species (Colwell 1986a, 1986b). This extreme specialization (“host monopolies” sensu Colwell 1986a, 1986b) became one of the most charismatic examples of mating rendezvous evolution—an evolutionary process in which host or habitat specificity may arise through frequency-dependent selection for finding mates (Futuyma and Moreno 1988). In the case of hummingbird flower mites, specialization to a particular host plant is assumed to increase chances to find mates. Over generations, host fidelity is expected to promote reproductive isolation and speciation (Colwell 1986a; Futuyma and Moreno 1988).

DNA barcoding, a molecular technique that involves sequencing of short (~600-bp) DNA fragments to identify species, is revealing an unprecedented cryptic diversity, especially in groups of organisms with limited taxonomic information (Hebert et al. 2004; Witt et al. 2006; Lee et al.

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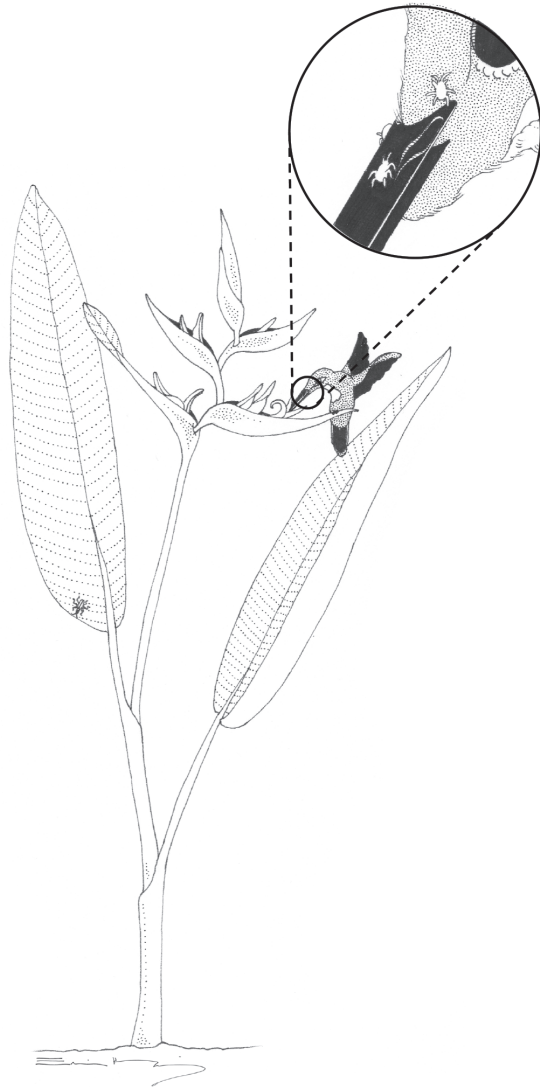


Figure 1: Hummingbird-pollinated flowers harbor a group of nectar robbers, the hummingbird flower mites (Ascidae: *Proctolaelaps*, *Rhinoseus*, and *Tropicoseus*). Mites spend the majority of their lives in or around the inflorescences of their host plants. To disperse to new plants, mites hitch rides in the nares of the hummingbirds that pollinate their host plants. Illustration by Erin K. Kuprewicz.

2017). Recent studies of mite taxonomy using DNA barcoding show that many well-established morphological species are cryptic species complexes (Schäffer et al. 2019; Young et al. 2019). It is possible that the original observation of extreme specialization that inspired the host monopolies and models supporting mating rendezvous evolution in hummingbird flower mites is an artifact of the limitations associated with morphological identification. In this study, we modified high-throughput DNA extraction techniques to obtain high-quality mite DNA. Our protocols

allowed us to obtain high-quality sequences with a 76% success rate (i.e., the percentage of high-quality sequences out of all sequences produced) and to keep vouchers of undamaged exoskeletons associated with DNA sequences.

As our study system, we used a community of hummingbird-pollinated plants and associated flower mites in a tropical rain forest in Costa Rica, Central America. The objectives of this study were (1) to identify the interactions among hummingbird flower mite species and their associations with their host plants and (2) to determine whether mite-plant associations follow the patterns of extreme specialization (i.e., the host monopolies assumed by the mating rendezvous hypothesis).

Methods

Study Site and Species

We performed this study at La Selva Biological Station (hereafter, La Selva), a tropical wet forest in the Caribbean lowlands of northeastern Costa Rica, Central America (10°26'N, 83°59'W). At La Selva, at least 14 species of plants are pollinated by hummingbirds (Stiles 1975; Maglianesi et al. 2015). Previous research based on morphology recorded 8–10 mite species at La Selva (Heyneman et al. 1991).

Molecular Identification of Flower Mite-Plant Interactions

We identified flower mite-plant interactions by collecting 489 flowers from all 14 known hummingbird-pollinated plant species flowering from May to October 2018 (mean flowers per plant species = 32.6, SD = 28.24; see sample size and species in table S1; tables S1, S2 are available online). A total of 10,654 mites were collected from the 489 flowers (mean mites per flower = 23.04, SD = 52.58), of which we selected a subsample to perform DNA barcode analyses on, to reconstruct flower mite-plant interactions. Subsamples were selected to ensure equivalent sampling across plant species. We extracted DNA from 1,928 mite specimens and amplified the cytochrome c oxidase I (COI) DNA barcode, a short mitochondrial sequence (~600 bp), which allowed us to delineate mite species (see DNA extraction, amplification and purification methods in supplement S1, available online). A total of 1,462 DNA samples produced viable sequences for further analyses. Sequences were aligned using MUSCLE (Edgar 2004; see sequence analysis methods in supplement S1). We performed a visual check of all of the sequences included in the alignment, removed gaps, and confirmed ambiguous bases. Additionally, we queried our aligned sequences against the National

Center for Biotechnology Information database via a blastn search to ensure that no contamination from foreign DNA was present (Altschul et al. 1990). Sequence alignments can also be visualized as a matrix of genetic distances, where each pair of sequences is represented by the percentage of bases from the residual that are identical, an automatic feature of the alignment tool in Geneious software (Kearse et al. 2012). These percentages were used to create a histogram of the frequency of genetic similarities among sequences in the alignment, allowing for the identification of a barcode gap. A barcode gap is determined as a gap in the percentage of genetic similarities that indicates the cutoff between intra- and interspecific similarity. This barcode gap was used to group sequences into molecular operational taxonomic units (MOTUs), which were used as our working hypothesis for species delimitation. We subsequently used morphological characters to assign each MOTU to a genus based on slide-mounted specimens (fig. 2). Using the alignment of 1,462 sequences, we created a rooted neighbor-joining tree (fig. 2B). As an out-group to root the tree, we used the CO1 sequence of an unidentified Canadian Ascidae species downloaded from GenBank (accession no. MN678094).

Statistical Analysis

To calculate the number of host plants used by each mite species and the number of mite genera and species present in each host plant, we assembled a qualitative (presence/absence) interaction matrix. The relative abundance of mite species in each host plant remains unknown because we used subsamples of mites collected in each flower to determine the diversity of mites interacting with hummingbird-pollinated plants.

To determine whether mites are isolated in host monopolies or whether mite species share hosts with congeners, generalist or specialist species, we performed a network structure analysis (R Core Team 2018; Dormann et al. 2020). The goal of this analysis is to quantify host plant use patterns, not to propose potential ecological processes associated with the described structure (Bastolla et al. 2009; Stouffer and Bascompte 2011; Qian and Akçay 2020). We calculated the levels of compartmentalization and nestedness using the package bipartite version 2.15 (R Core Team 2018; Dormann et al. 2020). Nestedness was calculated using the `nestedness()` function in the package bipartite, using 10,000 null models. This function returns a temperature value between 0 and 100, where 0 indicates perfect nestedness and 100 indicates a completely random matrix. Compartmentalization was determined with the `compart()` function in the bipartite package, which returns the number of compartments that can be identified in the matrix. A compartmentalized structure would suggest that most in-

teractions can be arranged along the main diagonal of the matrix, and mites tend to specialize on one or a few host plants as suggested by the mating rendezvous hypothesis. A nested structure would indicate that interactions can be sorted to fit cells below the main matrix diagonal. In this scenario the assemblage of flower mites at La Selva includes both generalist species and specialist species. This structure also implies that generalists share host plants with specialist mite species, and plants associated with a single mite species are usually the hosts of generalist mite species (Prado and Lewinsohn 2004; Bascompte and Jordano 2006; Guimarães et al. 2007). Data for all analyses have been deposited in the Dryad Digital Repository (<https://doi.org/10.5061/dryad.crjdfn35b>; Bizzarri et al. 2021), and the R scripts are available on Zenodo (<https://doi.org/10.5281/zenodo.5596752>).

Results

DNA barcodes revealed 18 hummingbird flower mite species distributed among 14 species of plants (fig. 3A). The histogram of the percentage of genetic similarities among the 1,462 aligned sequences allowed us to identify a barcode gap between 87% and 95% similarity (fig. 2A). This similarity cutoff was used to categorize sequences into 18 MOTUs. Following morphological identifications, we recorded 12 species of *Proctolaelaps*, one species of *Rhino-seius*, and three species of *Tropicoseius* (fig. 3A). We collected only immature stages for two species, which could not be identified to genus (fig. 3A). Mite species from the same genus can share the same host plant. In some cases as many as five species in the same genus can be found on the same host plant (fig. 3B; mean number of mite species per plant species = 3.4, SD = 1.84). In 10 plant species, we recorded two to four mite species sharing the same flower (mean number of mite species per flower = 1.32, SD = 0.62; table S2).

The mite-plant interaction matrix is significantly nested ($T = 15.75$, null model 3: $T = 21.05$, variance = 26.38, $P = .05$), with only one compartment. These results show that specialist mites share host plants with generalists, but plants interacting with one or a few mite species are usually hosts of generalist mites. Eight mite species were each associated with single host plant species but shared their hosts with generalist mites. Of these eight mite species, four are represented by 1 or 2 sequences (less than 0.1% of all sequences), indicating that they are extremely rare species. However, it is also possible that the rarity of these species could be an artifact of our sampling, meaning that there is a potential for a broader host plant use if more specimens were to be sequenced. The remaining four specialist species were not rare (6–43 sequences per species) and

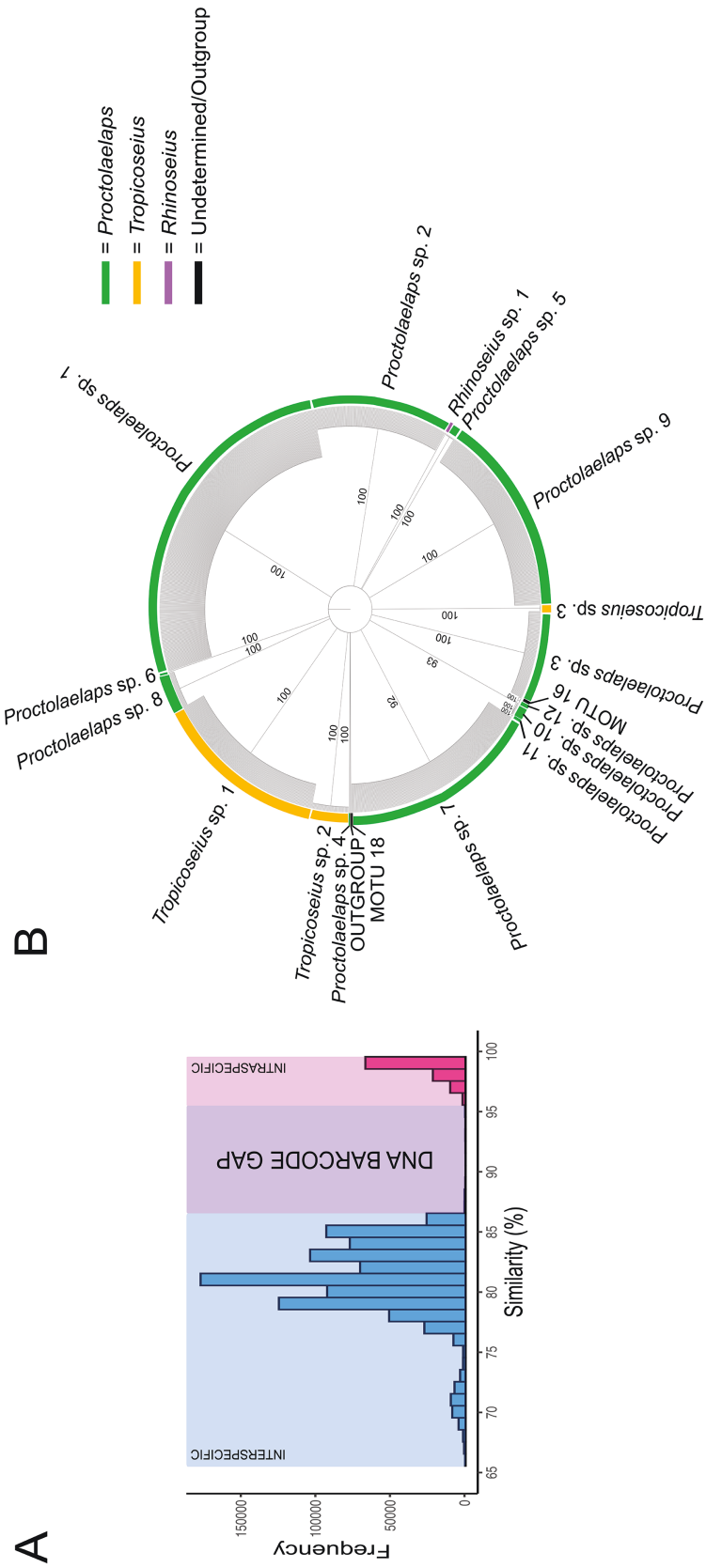
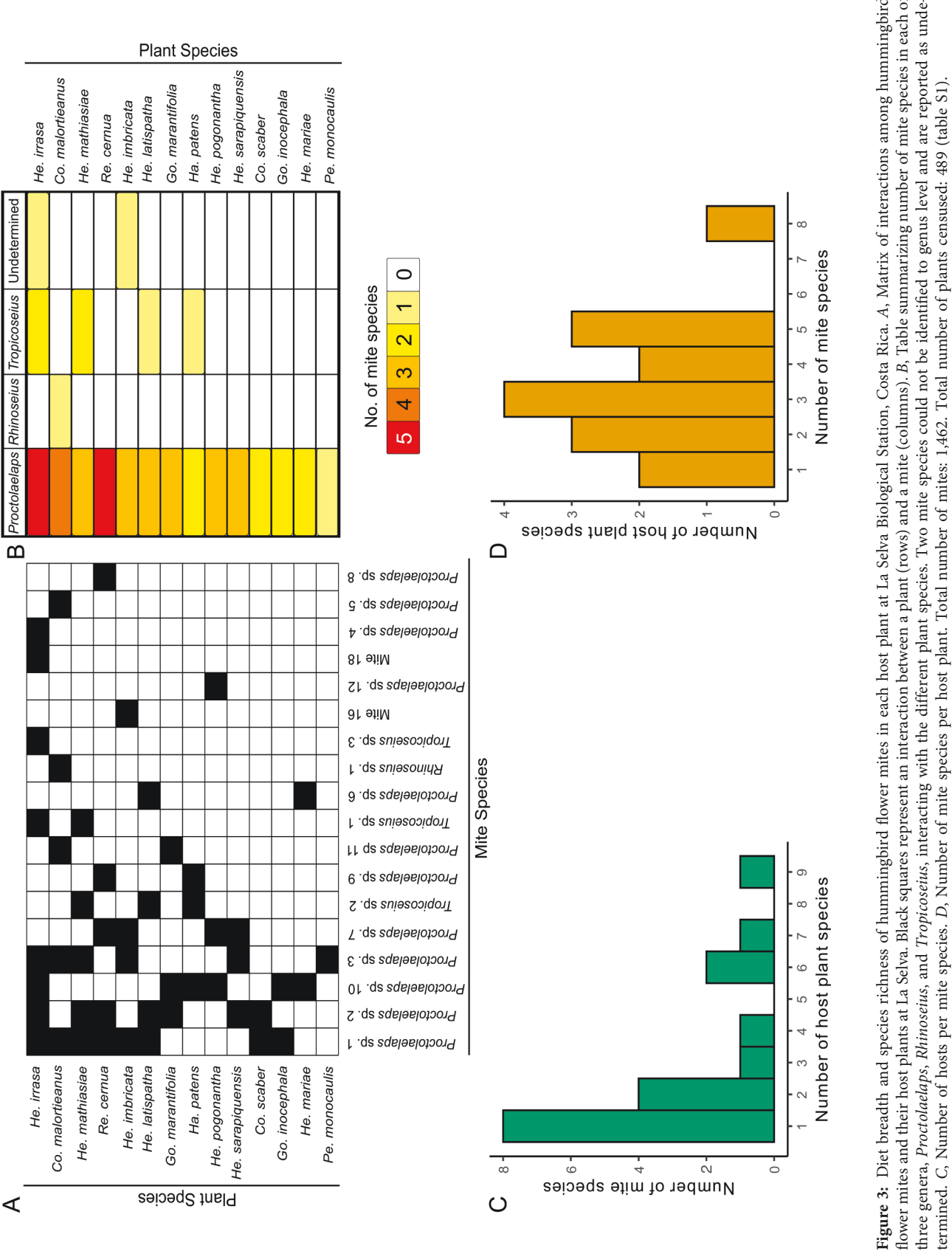


Figure 2: A, Frequency distribution of genetic similarities among mite cytochrome c oxidase I (COI) sequences. A clear DNA barcode gap is present between 87% and 95% similarity, representing the cutoff for species delineation. B, Delineation of hummingbird flower mite molecular operational taxonomic units (MOTUs) using COI sequences. The neighbor-joining tree includes bootstrap values (%) supporting the MOTU groups. An Ascidiae species was used as an out-group (GenBank accession no. MN678094).



were encountered on multiple plant individuals of the same plant species. We consider these four species true specialists, given that our sample contained multiple specimens belonging to these species (mean = 16.75, SD = 17.63). The remaining 10 mite species feed on two to eight hosts (fig. 3C). Five of the 14 plant species host a mix of generalist and specialist mite species (fig. 3D).

Discussion

According to the mating rendezvous hypothesis, the benefits of extreme specialization are twofold. First, it increases intraspecific encounters, increasing the probability to find a mate (Colwell 1986a, 1986b). Maximizing encounters with potential mates is particularly advantageous for organisms with limited dispersal ability and low population densities (Rohde 1979; Patton 1994). At La Selva both flower mites and hummingbirds are common; therefore, without any further evidence, there is no reason to assume that flower mites are dispersal limited or affected by Allee effects. Also the negative consequences of a complete reliance on a single or few host species (e.g., higher extinction risk or a reduction in fitness), most likely outweighs the benefits of ecological specialization driven by host fidelity (Futuyma and Moreno 1988).

The second putative benefit of extreme specialization in flower mites is that it promotes and maintains reproductive isolation by canalizing energy and gametes to intraspecific courtship and mating (Colwell 1986a). Reproductive isolation is the first step in speciation (Futuyma and Kirkpatrick 2017), and host fidelity is known to promote reproductive isolation among incipient species of other organisms (Feder et al. 1994; Funk et al. 2002; Soudi et al. 2015). However, there is no evidence that flower mites are incipient species or lack prezygotic reproductive barriers. Previous studies that used CO1 barcoding methods for species delineation and identification often use more stringent genetic similarity cutoffs than the one we utilize here (i.e., 2% in literature vs. 5% in this study). In contrast for parasitoid species complexes, differences among MOTUs can be as high as 11% (Chesters et al. 2012). Our cutoff was chosen to best reflect the frequency distribution of molecular similarities and the DNA barcode gap of this group of mites. We selected the most stringent cutoff that ensures accurate MOTU delimitation. The genetic distance between mite MOTUs in this study (87%–95%) is similar to distances reported for well-delimited species of mites (97.7%) and other arthropods (88%; Young et al. 2012; García-Robledo et al. 2016). This gives support to our hypothesis that mite MOTUs represent well-delimited species.

The general result of previous studies using DNA barcoding techniques is that species previously identified as

generalists are complexes of specialist species (Hebert et al. 2004; Witt et al. 2006; Smith et al. 2007; Chesters et al. 2012; Knee et al. 2012; Lee et al. 2017; Schaffer et al. 2019). DNA barcodes revealed the opposite pattern for hummingbird flower mites (i.e., mite species were assumed to be specialists), but we identified most species as host plant generalists. This surprising result stems from the initial assumption of extreme specialization, which motivated previous studies (Colwell 1986a, 1986b). Identifying closely related mite species using traditional taxonomy is challenging, even for expert taxonomists (O'Connor 2009). Our study is an example of how DNA barcoding can be used to overcome the “taxonomic impediment” in understudied taxa, such as phoretic mites (Cardoso et al. 2011).

DNA-based identifications of mite-plant interactions at La Selva show no evidence for host monopolies (Colwell 1986a, 1986b). Nested networks, such as the one described in this study, are commonplace among mutualistic and antagonistic assemblages, which are often the result of differences in abundances among interacting species and coevolutionary processes (Pires and Guimarães 2013) and most likely did not originate from mating rendezvous selection. A combination of ecological mechanisms, rather than sexual selection, is more likely to affect niche breadth and overlap of flower mites at La Selva (Vázquez et al. 2007; García et al. 2014).

Fitness differences among species can lead to coexistence by favoring invasibility of one species' habitat by another (Grainger et al. 2019). Interspecific competition among such species can ultimately lead to resource partitioning that favors species coexistence (Schoener 1974). Varying degrees of resource partitioning determine the degree of niche overlap or niche differentiation of two or more competing species. Resource partitioning among hummingbird flower mite species, rather than sexual selection, could be responsible for host plant use patterns among this guild of mites. Additionally, flower mites mainly consume and can even deplete nectar (Lara and Ornelas 2002), an essential resource for hummingbirds as well. Therefore, competition for nectar among mites and hummingbirds, in addition to competition for resources among mite species, could drive host plant use patterns of flower mites.

The mating rendezvous hypothesis provided for many years a captivating explanation for what was assumed to be a quintessential example of extreme ecological specialization in the tropics (Colwell 1986a, 1986b). DNA barcoding revealed that interactions in this group of mites resemble the more common case of generalist and specialist species sharing hosts. At La Selva each hummingbird-pollinated plant species is the rendezvous site of a particular assemblage of flower mites. The reason why flower mites group in particular subsets of host species still remains unknown, but most likely it is not sexual selection.

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Statement of Authorship

L.B. and C.G.-R. designed the study, L.B. collected the data, L.B. and C.S.B. developed molecular methods, L.B. conducted statistical analyses, L.B. and C.G.-R. led the writing of the manuscript, and all authors contributed to editing the manuscript.

Data and Code Availability

DNA sequences can be accessed on GenBank under accession numbers MW145544–MW147005. Data and code can be accessed at the Dryad Digital Repository (<https://doi.org/10.5061/dryad.crjdfn35b>; Bizzarri et al. 2021) and Zenodo (<https://doi.org/10.5281/zenodo.5596752>).

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