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ORIGINAL ARTICLE

A remarkable legion of guests: Diversity and host specificity of army ant symbionts

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Abstract
Tropical rainforests are among the most diverse biomes on Earth. While species inventories are far from complete for any tropical rainforest, even less is known about the intricate species interactions that form the basis of these ecological communities. One fascinating but poorly studied example are the symbiotic associations between army ants and their rich assemblages of parasitic arthropod guests. Hundreds of these guests, or myrmecophiles, have been taxonomically described. However, because previous work has mainly been based on haphazard collections from disjunct populations, it remains challenging to define species boundaries. We therefore know little about the species richness, abundance and host specificity of most guests in any given population, which is crucial to understand co-evolutionary and ecological dynamics. Here, we report a quantitative community survey of myrmecophiles parasitizing the six sympatric *Eciton* army ant species in a Costa Rican rainforest. Combining DNA barcoding with morphological identification of over 2,000 specimens, we discovered 62 species, including 49 beetles, 11 flies, one millipede and one silverfish. At least 14 of these species were new to science. Ecological network analysis revealed a clear signal of host partitioning, and each *Eciton* species was host to both specialists and generalists. These varying degrees in host specificities translated into a moderate level of network specificity, highlighting the system's level of biotic pluralism in terms of biodiversity and interaction diversity. By providing vouchered DNA barcodes for army

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ant guest species, this study provides a baseline for future work on co-evolutionary and ecological dynamics in these species-rich host–symbiont networks across the Neotropical realm.

KEYWORDS
biodiversity, community structure, cryptic species, ecological networks, host–symbiont networks, myrmecophiles

1 | INTRODUCTION

Habitat destruction is a major force of the dramatic biodiversity loss our planet is currently facing (United Nations Environment Programme, 2021). The most biodiverse terrestrial ecosystems—tropical forests—continue to be degraded at alarming rates, primarily due to the human demand for wood and agricultural land (Crowther et al., 2015; Hoang & Kanemoto, 2021). This loss of tropical forests is widely expected to cause a massive extinction of species at the local scale, and probably also the global scale (Alroy, 2017; Janzen & Hallwachs, 2017). The species-rich communities of tropical forests form complex interaction networks, for example between predators and prey (Gripenberg et al., 2019; Hoenle et al., 2019), hosts and parasites (Esser et al., 2016; Lopes et al., 2020), or plants and frugivores (Menke et al., 2011; Vidal et al., 2013). Identifying highly connected keystone species in these networks is crucial for efficient conservation measurements because their loss can have dramatic effects on community stability by causing the coextinction of many affiliated species (Dunne et al., 2002).

Army ants are keystone species in Neotropical communities because they are major consumers of arthropods (Hoenle et al., 2019; Kaspari & O'Donnell, 2003; Kaspari et al., 2011; Otis et al., 1986; Pérez-Espona, 2021; Powell, 2006, 2011; Powell & Clarke, 2004; Rettenmeyer et al., 1983; Vieira & Höfer, 1994). However, the ecological relevance of army ants goes beyond their impact as predators. Their large colonies sustain a diverse fauna of associated species, many of which depend on the presence of their army ant hosts (Gotwald Jr, 1995; Kronauer, 2020; Martínez et al., 2021; Rettenmeyer, 1961). Besides attracting conspicuous swarm followers such as specialized birds that feed on arthropods escaping from the ants (Gotwald Jr, 1995; Martínez et al., 2021), army ants also host a microcosm of invertebrates that take advantage of the abundant colony resources (Gotwald Jr, 1995; Kronauer, 2020; Rettenmeyer, 1961). These guests, or myrmecophiles, can be purely phoretic using the ants solely as dispersal agents, or they feed on the ants' refuse or prey as commensals. In some cases, myrmecophiles also prey directly on the ants and their brood (e.g., Akre, 1968; Akre & Rettenmeyer, 1966; Akre & Torgerson, 1969; Rettenmeyer, 1961). To avoid or withstand host attacks, many of the guests exhibit elaborate adaptations, such as chemical and gestalt mimicry of their host, as well as protective morphologies (von Beeren et al., 2018; Hölldobler & Wilson, 1990; Kistner, 1982; Parker, 2016; Parmentier, 2020; Figure 1). The interactions between army ants and their entourage of associates prominently illustrates the possible effect of local coextinction events, because the many obligate associates would probably face extinction if army ants disappeared in any given population (Boswell et al., 1998; Brown & Feener, 1998; Koh et al., 2004; Kronauer, 2020; Kumar & O'Donnell, 2007; Pérez-Espona, 2021).

Among social insects, the massive colonies of army ants host an exceptionally species-rich and taxonomically diverse guest fauna, including beetles, mites, spiders, silverfish, millipedes, flies and wasps (Gotwald Jr, 1995; Kronauer, 2020; Rettenmeyer, 1961). Carl Rettenmeyer and colleagues listed about 50 insect guests of the army ant *Eciton burchellii* (Westwood, 1842) that have been collected either in and around army ants' temporary nesting sites (bivouacs) or in colony emigrations (Rettenmeyer et al., 2011). This outstanding diversity might be explained by the area–diversity relationship of the theory of island biogeography (Darlington Jr, 1957; MacArthur & Wilson, 1967; Mittelbach, 2012)—a pervasive ecological pattern that is applicable to partially isolated ecosystems such as social insect colonies (Gotwald Jr, 1995; Hölldobler & Wilson, 1990; Kronauer & Pierce, 2011; Parmentier et al., 2020; Wilson, 1971). According to the theory (MacArthur & Wilson, 1967), large social insect colonies such as those of army ants are expected to harbour a high diversity of associated species, partly because of high microhabitat heterogeneity and low symbiont extinction rates (Gotwald Jr, 1995; Hölldobler & Wilson, 1990).

Besides large colony sizes, a defining feature of army ants is a nomadic lifestyle in which colonies regularly move to new hunting grounds (Gotwald Jr, 1995; Kronauer, 2020; Schneirla, 1971). During these emigrations, the guests travel along with the ants, permitting relatively easy collection (Gotwald Jr, 1995; Kronauer, 2020; Rettenmeyer, 1963; Figure 1). This access to otherwise hidden ant guests, along with their diversity, might partly explain why generations of researchers have been attracted to study army ant myrmecophiles (e.g., Borgmeier, 1961; Disney & Rettenmeyer, 2007; Kistner & Jacobson, 1990; Reichensperger, 1938; Rettenmeyer, 1961; Seevers, 1965; Tishechkin, 2007). Yet, studies have largely been confined to taxonomic work, with few behavioural observations and life history reports (e.g., Akre, 1968; Akre & Rettenmeyer, 1966; Akre & Torgerson, 1969; von Beeren et al., 2016a, 2016b, 2018; Disney & Rettenmeyer, 2007; Reichensperger, 1924; Rettenmeyer & Akre, 1966; Torgerson & Akre, 1969). A community-wide examination of army ant–myrmecophile interactions is still lacking, although information on prevalence and host specificity is crucial to understand the co-evolutionary and ecological dynamics in host–symbiont communities (Combes, 2005; Moore, 2002; Poulin et al., 2011; Schmid-Hempel, 2011), and to model and predict the coextinction risk of the associated symbiotic fauna if army ants disappeared (e.g., Dunn et al., 2009).

FIGURE 1 Examples of myrmecophiles participating in army ant colony emigrations. (a) Drop-shaped rove beetle *Vatesus* cf. *clypeatus* sp. 2 running amongst *Eciton hamatum* host workers (Costa Rica; photograph by D. Kronauer). (b) Histerid beetle *Nymphister kronaueri* hitchhiking in an *Eciton mexicanum* emigration by attaching between the ant's petiole and postpetiole (Costa Rica; photograph by M. Maruyama). (c) Two drop-shaped ptiliid beetles (*Cephaloplectus mus*) sitting on a prey ant being carried by two *Eciton vagans* workers (Venezuela; photograph by D. Kronauer). (d) Rove beetle *Proxenobius borgmeieri* running in an emigration column of *E. hamatum* (Costa Rica; photograph by D. Kronauer). (e) Phorid fly running in an emigration of *E. vagans* (Belize; photograph by A. Wild). (f) Ant-resembling (myrmecoid) *Ecitophya* rove beetle participating in an *Eciton burchellii* colony emigration (Peru; photograph by T. Komatsu)

First attempts to acquire such information have been made by compiling host records across different collection sites (e.g., von Beeren & Tishechkin, 2017; Ivens et al., 2016; Kistner, 1979; Kistner & Jacobson, 1990; Rettenmeyer & Akre, 1966; Seevers & Dybas, 1943), which is a common approach to estimating a symbiont's host range (Glasier et al., 2018; Parmentier et al., 2020; Poulin & Morand, 2014). However, these composite lists suffer from several shortcomings (Poulin et al., 2011; Thompson, 2005). A symbiont's host preference can greatly vary between populations (Krasnov et al., 2004; Poulin et al., 2011; Poulin & Morand, 2014; Thompson, 2005), and lumping data across populations often denotes a symbiont as a host generalist, while in fact it is a host specialist at the population level (Poulin et al., 2011; Thompson, 2005). Furthermore, such lists often suffer from sampling bias because symbionts of more abundant host species are generally overrepresented (Poulin & Morand, 2014). We have previously reported the first community-based host specificities of army ant guests, but these studies focused on specific taxonomic groups (von Beeren, Maruyama, & Kronauer, 2016a, 2016b; von Beeren & Tishechkin, 2017; Tishechkin et al., 2017). As a result, community structure and interaction specificities at the level of the entire army ant–myrmecophile community remain unknown.

Here, we present a comprehensive study encompassing an entire army ant–myrmecophile community. Over the course of four years, we systematically collected myrmecophiles from 70 colonies across all six *Eciton* Latreille, 1804 army ant species occurring at La Selva Biological Station, Costa Rica. Because myrmecophile identification was challenging due to the presence of morphologically extremely similar species (von Beeren et al., 2016a, 2016b; Tishechkin et al., 2017), we used a molecular approach by supplementing morphological identifications with extensive DNA barcoding to define species boundaries. We then studied community structure as well as host specificity at the species- and at the network-level using weighted ecological network analyses (Blüthgen et al., 2008; Ings et al., 2009; Ivens et al., 2016; Poulin, 2010).

2 | METHODS

2.1 | Collection protocol and research permits

The present study is a synopsis of our work on *Eciton* myrmecophiles at La Selva Biological Station (LSBS) during the years 2013, 2014, 2015 and 2017, which also includes previously published

TABLE 1 Collection information and network specificity parameters

Army ants	Colonies	Emigrations	No. of specimens	Total no. of spp.	No. of beetle spp.	No. of fly spp.	No. of millipede spp.	No. of silverfish spp.	e^H	e^H_{rare} ±SD	*d'*	Inc.
E. burchellii	13	27	739 (554)	36	28	7	0	1	21.65	14.21 ± 1.86	0.45	117
E. dulcium	14	14	346 (341)	13	9	3	0	1	9.65	8.58 ± 0.84	0.53	75
E. hamatum	15	16	493 (450)	22	14	7	0	1	14.69	11.32 ± 1.27	0.50	111
E. lucanoides	4	4	151 (138)	19	14	4	0	1	16.32	16.32 ± 0.00	0.38	33
E. mexicanum	14	16	412 (375)	13	7	4	1	1	9.90	8.64 ± 0.75	0.48	84
E. vagans	10	11	280 (255)	11	6	4	0	1	8.85	8.12 ± 0.63	0.49	62

Note: Given is the number of colonies from which myrmecophiles were collected for network analyses. Because some colonies were sampled more than once, the number of emigrations is sometimes higher than the number of colonies. Number of specimens describes the total number of myrmecophiles used for morphological and genetic species identifications, which contains specimens from additional colonies including collections from army ant raids and refuse deposits. Numbers in parentheses give the total numbers of myrmecophile specimens used for network analysis. Myrmecophile diversity is given as total number of species, the total number of species separated per arthropod order, as effective Shannon diversity (e^H; Jost, 2006), and as the mean of 100 rarefied e^H (e^H_{rare}). Exclusiveness of myrmecophiles is given as Kullback–Leibler distance (*d'*; Blüthgen et al., 2006). Network incidences (Inc.) give the total number of occurrences of different myrmecophile species across all colonies of an army ant species.

morphological descriptions and DNA sequence data (von Beeren et al., 2016a, 2016b, 2018; von Beeren & Tishechkin, 2017; Tishechkin et al., 2017; Table S1). During a total of 7 months, we searched for army ant colony emigrations, primarily between 8 p.m. and 3 a.m., by walking the trails at LSBS—a lowland Costa Rican tropical rainforest (GPS data: 10°25′19.2″N, 84°0′54″W; 35–137 m a.s.l.). We covered an area of ~11 km^2 and collected myrmecophiles from all locally occurring *Eciton* species (Table 1): *E. burchellii* (subspecies *E. burchellii foreli* Mayr, 1886), *Eciton dulcium* Forel, 1912, *E. hamatum* (Fabricius, 1782), *E. lucanoides* Emery, 1894, *E. mexicanum* Roger, 1863 and *E. vagans* (Olivier, 1792).

Upon encountering an emigration, we observed it until the last ants and myrmecophiles had passed, usually for several hours, and collected ants and as many myrmecophiles as possible using aspirators and forceps. In cases of slight and repeated disturbance, the ants sometimes stopped emigrating for a short period of time (~5–30 min) before continuing. During this phase it is best to not further disturb the ants, because otherwise they establish alternative emigration routes. This happened rarely in *E. burchellii*, *E. hamatum*, and *E. lucanoides*, but regularly in the other *Eciton* species. We thus collected myrmecophiles in teams of at least two people, with a maximum of four people, to also cover alternative emigration routes. We remained at the collection sites for about 30–60 min after colony emigrations had concluded to collect any remaining trail-following army ant myrmecophiles. Specimens attached to large workers were collected with forceps together with the ants. Myrmecophiles running in the emigration column independently, as well as those attached to small workers, were collected with custom-built aspirators (inner tube diameter 8–10 mm).

Within 2 hrs of collection, we added absolute ethanol to collection tubes (50-ml Falcon vials) to preserve army ants and myrmecophiles for morphological and genetic analyses. In total, we collected myrmecophiles from 70 colonies (range: four to 15 colonies per species, Table 1). Samples were then transferred to The Rockefeller University and later to the TU Darmstadt, where we stored them at −30°C until further processing. Collection permits, export permits and research permits were issued by the "Ministry of the Environment, Energy and Technology" and the "National Commission for Biodiversity Management" (MINAET; permit numbers: 192–2012-SINAC, R-009–2014-OT-CONAGEBIO and R- 007–2017-OT-CONAGEBIO).

For reasons of feasibility, we restricted our systematic community analysis to myrmecophile species participating in army ant colony emigrations (Figure 1). Some of the species participating in emigrations can also be found in raids and/or refuse deposits. Species that also participate in raids include myrmecoid *Ecitophya* and *Ecitomorpha* rove beetles, as well as the ptiliid *Cephaloplectus mus*. In addition to standardized collections from emigrations, we haphazardly collected myrmecophile specimens from *Eciton* raids and refuse deposits. While these specimens were not examined systematically, we integrated some of them in the current analysis for the sole purpose of increasing sample sizes in genetic and morphological assessments of species boundaries (see Table S1 for a full list). We excluded mites from the current study due to the difficulties of collecting them, their

high diversity and their complicated taxonomy (e.g., Berghoff et al., 2009). Many mites are diminutive and attach to various body parts of army ant workers (Gotwald Jr, 1995; Rettenmeyer, 1963). Conducting a reliable community analysis of mite diversity and host specificity would have required a careful inspection of army ant bodies of hundreds of workers per colony (e.g., Berghoff et al., 2009), followed by a challenging and time-consuming identification process of mite taxa. This would have been beyond the available resources for this study. However, with the vouchered material we hope that a community-wide study on mite diversity and host specificity can eventually be included in future work. Furthermore, we did not purposely collect aerial parasitoids hovering over the army ants (Brown & Feener, 1998) because our search was focused on the marching ants and their associates on the ground.

Initially, we followed and intentionally resampled a few colonies, mostly of *E. burchellii*. This is the species with the largest colonies and the highest ant traffic, making it particularly challenging to spot and collect myrmecophiles (Table 1; Table S1). This resampling indicated that our approach was efficient in collecting most myrmecophile specimens occurring in a colony from a single colony emigration (see Table S1). For example, we collected 24 adult and three larval *Vatesus* cf. *clypeatus* sp. 2 specimens from the first observed emigration of the colony EB03 (Table S1). On the next day, we resampled guests from the emigration of the same colony and collected only four additional *Vatesus* adults and no *Vatesus* larvae. However, it is impossible to collect all myrmecophiles from a colony with certainty, and we almost certainly missed myrmecophiles in any given colony emigration, especially in those that we did not observe from the very beginning. Nonetheless, our resampling data indicate that, even in *E. burchellii*, a thorough representation of myrmecophiles can be acquired from a single colony emigration.

Because army ant colonies are nomadic, there is a risk of unintentionally resampling the same colony on different days (see also von Beeren et al., 2016a; Hoenle et al., 2019). Such events would represent pseudoreplicates in our network analyses of host–symbiont interactions (see below). To minimize resampling, we omitted colony emigrations of the same species within a period of 1 week in proximity (~200 m radius) of the last sampling spot. While we cannot exclude that resampling happened, we do expect that myrmecophile collections mostly covered different army ant colonies because sampling took place over four years in a relatively large collection area with high army ant colony density (O'Donnell et al., 2007).

2.2 | Species identification

The faunistic diversity of army ant myrmecophiles, together with deficiencies in taxonomic species descriptions, caused problems in species identifications (von Beeren et al., 2016a, 2016b, 2018) and we therefore implemented DNA barcoding, a technique used routinely to detect species boundaries in difficult taxonomic groups (Hajibabaei et al., 2006; Hebert et al., 2004; Janzen et al., 2017; Pečnikar & Buzan, 2014).

To streamline our identification process, we used the following three-step protocol. We first applied a morphological prescreening by sorting the collected myrmecophiles per colony to morphospecies, which we defined as morphologically similar specimens that were indistinguishable to us, even with the help of the latest species keys for the group. As a second step, we used, if available, at least five specimens per morphospecies and colony for DNA barcoding. Initially we had difficulties distinguishing phorid fly species, and we thus used, if available, at least 10 phorid fly specimens per colony for DNA barcoding. This sampling protocol implied that we did not comprehensively collect abundance data of myrmecophiles (number of specimens per colony) in the present study, even though such data were previously reported for certain taxa (von Beeren et al., 2016a, 2018; Tishechkin et al., 2017). We did not incorporate abundance data in the present work because genetic analyses were necessary to distinguish cryptic taxa (e.g., in certain phorid flies; see Results), which would have required barcoding hundreds of specimens per colony. However, we do provide abundance data for those species where fewer than five specimens were collected from a colony.

As a third step, we compared our morphological identifications with genetic clustering results. In most cases the two approaches agreed on the determination of species boundaries. In a few cases, however, contentious results arose as two or more genetic clusters could not be distinguished morphologically. We tried to resolve such controversies by acquiring both additional genetic information from nuclear gene loci and additional morphological characters. If morphological inspection and nuclear gene data disagreed with mitochondrial gene data, we invoked the morphological species concept and lumped specimens of distinct *cytochrome oxidase I* (*COI*) gene clusters into a single species. A set of specimens of each species was finally sent to the following taxonomic experts for verification of species identifications: Histeridae: A.K.T.; Staphylinidae: M.M., Alexey Solodovnikov, Mariana Chani-Posse & Taro Eldredge; Ptiliidae: Mikael Sörensson & W.E.H.; Hydrophilidae: Emmanuel Arriaga Varela & Martin Fikáček; Phoridae: B.V.B. & J.M.H.; Nicoletiidae: Luis Mendes; Pyrgodesmidae: Thomas Wesener. References to species keys of each group are given in Table S1. We used the species key for Costa Rican ants of Longino (2010) to identify army ant species.

We extracted DNA from 2,432 myrmecophile specimens using Qiagen DNeasy Tissue Kits, either for single extractions or for 96-well plates. We followed the standard protocol with two exceptions: specimens were not homogenized but kept intact and protein digestion was shortened to 2–3 hrs. Except for a few larval specimens and some phorid flies, this procedure preserved specimens in good conditions for later morphological examination. DNA extracts were then transferred to freezers at −30°C at the TU Darmstadt to serve as DNA vouchers (contact C.v.B. for access). After the procedure we stored the specimens in absolute ethanol until further morphological inspections.

For all DNA extracts, we tried to amplify the classical animal DNA barcode *COI* in standard polymerase chain reactions (PCRs). PCRs were set up as described previously (von Beeren et al., 2016a, 2016b; Hoenle et al., 2019). For each taxonomic group, we adjusted PCRs

by using various published and custom PCR primers and optimizing annealing temperatures (see Tables S2 and S3). Purification and sequencing of PCR products were outsourced to Macrogen USA and to Macrogen Europe. PCR amplicons were sequenced in forward and reverse directions using Sanger sequencing, which allowed us to link each DNA barcode to a voucher specimen. In cases of low-quality reads, PCR settings were adjusted, and sequencing was repeated.

For sequence analyses we used the laboratory information management system GENEIOUS PRIME programmed by BIOMATTERS (version 2020.1; https://www.geneious.com including the plugin "biocode"; version 3.0.7; Parker et al., 2012). This included assembly of forward and reverse sequences, sequence trimming, sequence editing, sequence alignment using the MUSCLE algorithm (Edgar, 2004), and clustering analyses. We performed several quality checks with the resulting consensus sequences. Sequences with stop codons in the *COI* alignment were omitted from further analyses as they probably represented nuclear mitochondrial pseudogenes. We compared barcoding results to morphological identifications to detect and omit apparently erroneous sequences due to contamination or pipetting errors (<1% of DNA barcodes). Final consensus sequences were deposited at the Barcode of Life Database systems (BOLD; GenBank accession numbers are given in Table S1).

In a few cases, *COI* analyses resulted in distinct genetic clusters that we morphologically identified as the same species. In such cases we additionally analysed portions of the nuclear gene *wingless* (*wg*) for a subset of specimens with different *COI* haplotypes (Table S1). Congruency in clustering analyses of mitochondrial sequences (*COI*) and nuclear sequences (*wg*), meaning that specimens from distinct *COI* clusters had distinct *wg* alleles, was interpreted as support for the coexistence of distinct species. In beetles, specimens of these candidate species were anatomically inspected in more detail to search for diagnostic morphological characters (e.g., aedeagi dissections: von Beeren et al., 2016a, 2016b). In phorid flies, we relied on genetic species assessment, and a more in-depth morphological analysis remains for future work. In contrast, discordance of genetic clustering, meaning divergent *COI* clusters shared the same *wg* sequences, was interpreted as support for the presence of a single species (see also von Beeren et al., 2015, 2016b).

As a consistent amplification of high-quality sequences of the full *wg* fragment targeted by the commonly used *wg* insect primers wg550F and wgAbrZ (Wild & Maddison, 2008) failed in phorid flies, we decided to analyse two shorter, nonoverlapping sequences of *wg*. Those two portions were located at the start and at the end of the full fragment and produced high-quality signals. The excluded portion in the middle part of the full fragment included an intron, which showed low sequence quality scores in many specimens. We denote these two portions here as *wingless* gene fragment I and *wingless* gene fragment II (for primers see Tables S2 and S3). In previous work we additionally analysed a portion of the *carbamoyl-phosphate synthetase II* gene of the multidomain enzyme CAD (*CAD*) to determine species boundaries in the genera *Ecitophya*, *Ecitomorpha*, *Vatesus* and *Tetradonia* (von Beeren et al., 2016a, 2016b, 2018).

To identify distinct genetic units in myrmecophile DNA barcodes, we applied the standardized sequence clustering algorithm RESL, which is implemented in the BOLD systems (Ratnasingham & Hebert, 2013). In short, RESL clusters uncorrected pairwise distances (p-distances) into genetic units based on sequence similarity and by setting thresholds for operational intracluster units and intercluster units. BOLD systems then designate RESL-based "Barcode Index Numbers" (abbreviated as BINs), which define distinct genetic units in the entire BOLD systems database. One of the main advantages of BIN numbers is that they provide a standard analytical tool for researchers to identify molecular taxonomic units (MOTUs) in a data set, without each study defining their own sequence similarity threshold for MOTUs. BINs thus represented our primary tool to help identify species based on molecular data. To visually represent clustering of genetic data, we additionally generated random accelerated maximum likelihood (RAxML) trees (Stamatakis, 2014) based on the GTR GAMMA nucleotide substitution model using the RAxML plugin for GENEIOUS PRIME (version 2020.2.1). RAxML tree analysis also allowed us to include *COI* sequences of less than 300 bp ($N = 7$ specimens; Table S1) in the molecular identification process, even though sequences of this length are not assigned to a BIN by the RESL algorithm (Ratnasingham & Hebert, 2013). All of these short sequences showed a 100% match to other sequences in the data set that were assigned to a BIN. The RAxML trees were rooted using the following outgroups: *Saprinus semistriatus* (Scriba, 1790) (Histeridae, GenBank accession no.: BCFOR076-15), *Melanderomyia kahli* Kessel, 1960 (Platypezidae, GenBank accession no.: GBDP18672-15), *Hydraena pensylvanica* Kiesenwetter, 1849 (Ptiliidae, GenBank accession no.: BARSA189-15), *Cephaloplectus mus* Mann, 1926 (Ptiliidae, GenBank accession no.: MW128659), *Vatesus* sp. (Staphylinidae, GenBank accession no.: MW471662) and *Apocephalus* Coquillett, 1901 sp. (Phoridae, GenBank accession nos.: *wg* gene fragment I: MW439321; *wg* gene fragment II: MW439320).

2.3 | Deposition of specimens, images and DNA extracts

We deposited 379 specimens of 47 species at 15 museum collections. All other specimens and all DNA extracts are currently stored at −30°C in C.v.B.'s personal collection at the TU Darmstadt Insect Collection (contact C.v.B. for access). Details on specimen depositories are given in Table S1 and on the BOLD website (http://www.boldsystems.org/).

In addition to specimen deposition, we uploaded 2,206 focus-stacked voucher images of 497 myrmecophile specimens to the BOLD systems (Table S1). Images were taken with three different setups. One consisted of a Leica Z16 APO stereomicroscope equipped with a Leica DFC450 camera and a light dome using the processing software LEICA APPLICATION SUITE (version 4). A second, custom-built setup consisted of a Canon EOS 7D camera equipped with a Canon MP-E 65-mm 1–5× macro lens. The camera was mounted on a COGNISYS StackShot macro rail (Extended Set) and lighting was adjusted using three external flashlights and a custom-built light dome. A Zeiss stage micrometer was used as scale. The third setup consisted of a

Keyence VHX-5000 digital microscope (Keyence Deutschland) using the VH-Z50L lens. We used HELICON REMOTE (version 3.9.10) for automated imaging and HELICON FOCUS (version 7.6.1) for focus-stacking.

2.4 | Network statistics

We analysed the diversity and interaction specificity of the army ant–myrmecophile community using standardized analytical metrics provided by ecological network theory (Blüthgen, 2010; Ivens et al., 2016). Overall, 2,113 myrmecophile specimens were included in the evaluation of species- and network-level specificity (Tables 1 and 2; Table S1). The network analysis of the present study was based on a quantitative bipartite interaction matrix (Table S1). An "incidence" was defined as a myrmecophile species occurring in a colony of an army ant species. The "link strength" between an army ant species and a myrmecophile species represented the number of times a given myrmecophile species was detected in different colonies of that host. In other words, the "link strength" summarizes the incidences between an army ant species and a myrmecophile species, serving as a measure of colony infestation frequency in the population. This means that specimens of the same species collected from the same colony were only represented by a single incidence count. The network therefore contained incidence data of spatiotemporally independent collection events, which yields a conservative estimate of host specificity (Blüthgen et al., 2006; Hoenle et al., 2019). This quantification is important because unweighted networks often underestimate interaction specificity (Blüthgen, 2010; Blüthgen et al., 2006; Hoenle et al., 2019). For instance, when a myrmecophile associates primarily with one host species but is occasionally found with another host, relying solely on presence/absence data would underestimate its true level of host specificity (Blüthgen, 2010; Blüthgen et al., 2006; Hoenle et al., 2019; Poulin et al., 2011). All network statistics were analysed in R (R Core Team, 2020) using the package "bipartite" (Dormann et al., 2008; version 2.08).

Based on the bipartite network, we analysed the network-level specificity and the species-level specificity by calculating the two-dimensional Shannon entropy H_2' and the Kullback-Leibler distance d', respectively. These weighted metrics have several benefits over unweighted network analysis (Blüthgen, 2010; Blüthgen et al., 2006). Values for H_2' and d' are normalized relative to minimum and maximum possible values, thus ranging from 0 (lowest interaction specificity) to 1 (highest interaction specificity) (Blüthgen et al., 2006). For instance, from the host perspective, a low d' value (close to zero) means that the myrmecophile fauna of that host is relatively unspecific (nonexclusive myrmecophile fauna), and a value close to one means that a host's myrmecophile fauna is entirely different from the myrmecophile fauna of other hosts (exclusive myrmecophile fauna). From the perspective of a myrmecophile, high d' values are characteristic of host-specific species and of species that were collected from rare hosts. This is because the metric d' takes into account the abundance of myrmecophile species as well as the abundance of host species by considering the number of interactions of a particular myrmecophile relative to the total number of interactions of its hosts (for details see Blüthgen et al., 2006). One example of a rare species with a relatively high d' value is the histerid *Aphanister* sp. 1 ($d' = 0.27$; $N = 1$ specimen), which was collected from the rare host *E. lucanoides* (Tables 1 and 2). Low d' values are found in host generalists as well as in rare species that are associated with common hosts. For instance, only one specimen of the histerid beetle *Aemulister hirsuta* was collected in one colony of the more abundant host *E. mexicanum*. Accordingly, its host specificity was comparably low ($d' = 0.07$; Table S1). We tested the metric H_2' against null models based on 10,000 randomized networks using the Patefield algorithm (Blüthgen et al., 2006; Patefield, 1981).

Additionally, we calculated the effective Shannon diversity of interaction partners per species (e^H; Jost, 2006). This metric takes the richness and evenness of interactions into account and thus suitably characterizes link strengths for each species (Blüthgen, 2010). Furthermore, we calculated the rarefied Shannon diversity ($e^H{}_{rare}$) based on 100 permutations of 33 incidences, representing the lowest incidence number of a host species in the present network (*E. lucanoides*; Table 1). The rarefied metric $e^H{}_{rare}$ improves comparability and accounts for variation in sample sizes between army ant species (Table 1). Note that myrmecophile rarity is not considered in this metric, and assessment of host specificity needs to be related to sample size when referring to e^H. For instance, the histerid beetle *Aemulister hirsuta* had an e^H value of 1.00 host species, which could indicate high host specificity. However, because only one individual was collected, the actual host range and host specificity remains elusive for this species. Table 2 provides a summary of myrmecophile sample sizes for each species, allowing the reader to evaluate host specificity in the context of myrmecophile rarity.

Besides examining specificity and diversity, we used two complementary approaches to explore the extent to which myrmecophiles partition the available *Eciton* host niche space. First, we measured the degree of network modularity (metric Q calculated by the QuanBiMo algorithm; Dormann & Strauss, 2014). This metric quantifies to what extent data support the division of a network into modules (Dormann & Strauss, 2014). Modules are characterized by a high density of within-module links and few to no between-module links. The metric Q is normalized and ranges from 0 (no more links within modules than expected by chance) to 1 (perfectly modular networks). We tested Q against randomized null models as described previously (Hoenle et al., 2019; Schleuning et al., 2014). Second, we tested whether myrmecophile faunas differed between army ant species pairs by comparing H_2' values of each army ant species pair to 1,000 randomized networks. Species pairs consisted of two army ant species and all their associated myrmecophiles (see also Wehner et al., 2018).

The literature on host associations (e.g., Ivens et al., 2016; Kistner, 1982; Kistner & Jacobson, 1990) and additional collections of army ant associates by us indicate that the selected *Eciton*–myrmecophile network represents an almost closed network (or a well-defined module) within the entire army ant–myrmecophile network at LSBS. In other words, the myrmecophiles of *Eciton* army ants generally do not occur in colonies of other army ant genera. This is

TABLE 2 Overview of *Eciton*-associated myrmecophiles and their host specificity

Order	Family	Myrmecophile species	*N*	Inc.	Hosts	e^{H}_{hosts}	*d'*
Coleoptera	Histeridae	*Aemulister hirsuta* (Helava in Helava et al., 1985)	1	1	1 EM	1.00	0.07
		Aphanister sp. 1 Reichensperger, 1933 *	1	1	1 EL	1.00	0.27
		Cheilister cf. *lucidulus* Reichensperger, 1924	1	1	1 EB	1.00	0.00
		Clientister Reichensperger, 1935 sp. 1 *	1	1	1 EB	1.00	0.00
		Psalidister furcatus Reichensperger, 1924	1	1	1 EB	1.00	0.00
		Colonides cf. *collegii* Reichensperger, 1923	1	1	1 EB	1.00	0.00
		Daptesister pilosus Helava in Helava et al. 1985	15	4	1 ED	1.00	0.36
		Ecclisister costaericae Reichensperger, 1935 *	9	2	1 EB	1.00	0.14
		Euclasea Lewis, 1888 sp. 1 *	2	1	1 EB	1.00	0.00
		Euxenister caroli Reichensperger, 1923	4	2	1 EB	1.00	0.14
		Euxenister wheeleri Mann, 1925	16	7	1 EH	1.00	0.34
		Nymphister kronaueri von Beeren & Tishechkin, 2017 *	58	10	1 EM	1.00	0.45
		Nymphister monotonus (Reichensperger, 1938)	1	1	1 EH	1.00	0.01
		Nymphister simplicissimus Reichensperger, 1938	1	1	1 EB	1.00	0.00
		Sternocoelopsis cf. *nevermanni* Reichensperger, 1932	4	2	2 EB, EL	2.00	0.13
		Sternocoelopsis cf. *veselyi* Reichensperger, 1923	2	1	1 EB	1.00	0.00
		Symphilister cf. *hamati* Reichensperger, 1929	1	1	1 EB	1.00	0.00
	Hydrophilidae	*Sacosternum* aff. *lebbinorum* Fikáček & Short 2010 *	4	3	1 EB	1.00	0.21
	Ptiliidae	*Cephaloplectus mus* Mann, 1926	93	23	6 EB, ED, EH, EL, EM, EV	3.84	0.10
		Limulodes Matthews, 1867 sp. 1	5	2	1 EV	1.00	0.27
		Limulodes Matthews, 1867 sp. 2	14	7	2 ED, EM	1.82	0.28
		Limulodes Matthews, 1867 sp. 3	22	9	3 EB, ED, EL	3.00	0.22
		Limulodes Matthews, 1867 sp. 4	3	3	3 EB, ED, EM	3.00	0.03
		Nossidium Erichson, 1845 sp. 1	1	1	1 EB	1.00	0.00
	Staphylinidae	aff. *Tetradonia* Wasmann, 1894 sp. 1	5	1	1 ED	1.00	0.10
		Campbellia lucanoides (Campbell, 1973)	12	1	1 EL	1.00	0.27
		Ecitopora Wasmann, 1887 sp. 1	5	2	2 EH, EL	2.00	0.14
		Ecitopora Wasmann, 1887 sp. 2	1	1	1 EB	1.00	0.00
		Ecitomedon harpax Reichensperger, 1938	1	1	1 EV	1.00	0.13
		Ecitomorpha cf. *breviceps* Reichensperger, 1933	9	6	1 EB	1.00	0.31
		Ecitomorpha cf. *nevermanni* Reichensperger 1935	18	6	1 EB	1.00	0.31
		Ecitophya gracillima Mann, 1925	22	11	1 EH	1.00	0.38
		Ecitophya simulans (Wasmann, 1889)	46	9	1 EB	1.00	0.35
		Myrmedonota Cameron, 1920 sp. 1 *	29	3	1 EL	1.00	0.48
		Myrmedonota Cameron, 1920 sp. 2	4	2	2 EH, EL	2.00	0.14
		Quedius Stephens, 1832 (*Pridonius* Blackwelder 1952) sp. 1	1	1	1 EB	1.00	0.00
		Pseudofalagonia cf. *crassiventris* (Sharp, 1883)	5	2	1 ED	1.00	0.24
		Proxenobius borgmeieri Seevers, 1965	29	8	1 EH	1.00	0.35
		False Lomechusini sp. 1	1	1	1 EH	1.00	0.01
		False Lomechusini sp. 2	1	1	1 EB	1.00	0.00

(Continues)

TABLE 2 (Continued)

Order	Family	Myrmecophile species	N	Inc.	Hosts	e^{H}_{hosts}	d'
		Tetradonia cf. *marginalis* Reichensperger, 1935	133	30	4 EB, EH, EL, EV	3.04	0.18
		Tetradonia laselvensis Maruyama & von Beeren, 2016 *	13	9	3 EB, EH,EL	2.70	0.18
		Tetradonia laticeps Jacobson & Kistner, 1998	151	28	6 EB, ED, EH, EL, EM, EV	4.92	0.08
		Tetradonia lizonae von Beeren & Maruyama, 2016 *	101	15	2 EB, EH	1.28	0.35
		Tetradonia tikalensis Jacobson & Kistner, 1998	9	5	3 EB, EH, EL	2.87	0.18
		Vatesus aff. *goianus* Borgmeier, 1961 *	151	26	2 ED, EM	1.99	0.38
		Vatesus cf. *clypeatus* sp. 1 (Wasmann, 1887) *	57	10	1 EV	1.00	0.52
		Vatesus cf. *clypeatus* sp. 2 (Wasmann, 1887) *	345	29	3 EB, EH, EL	2.26	0.24
		Vatesus cf. *clypeatus* sp. 3 (Wasmann, 1887) *	5	1	1 EL	1.00	0.27
Diplopoda	Stylodesmidae	*Calymmodesmus montanus* Loomis, 1964	3	1	1 EM	1.00	0.07
Diptera	Phoridae	*Apocephalus* Coquillett, 1901 sp. 1	1	1	1 EH	1.00	0.01
		Dohrniphora ecitophila Borgmeier, 1960	19	11	3 ED, EM, EV	2.70	0.23
		Ecitophora bruchi Schmitz, 1923	79	11	1 EM	1.00	0.46
		Ecitophora cf. *comes* sp. 1 Schmitz, 1914 *	105	39	6 EB, ED, EH, EL, EM, EV	5.51	0.02
		Ecitophora cf. *comes* sp. 2 Schmitz, 1914 *	18	6	3 EB, EH, EL	2.38	0.17
		Ecitophora cf. *comes* sp. 3 Schmitz, 1914 *	12	6	3 EB, EH, EL	1.57	0.25
		Ecitophora halterata (Borgmeier 1936)	3	3	2 EB, EH	1.89	0.07
		Ecitophora pilosula Borgmeier, 1960	91	13	3 EB, ED, EH	1.31	0.43
		Ecitophora varians Borgmeier, 1960	50	10	1 EV	1.00	0.52
		Ecituncula tarsalis Borgmeier, 1925	26	7	3 EB, EH, EL	2.60	0.19
		Thalloptera fuscipalpis (Schmitz, 1923)	64	22	4 EB, EH, EM, EV	2.92	0.24
Thysanura	Nicoletiidae	*Trichatelura manni* (Caudell, 1925)	227	57	6 EB, ED, EH, EL, EM, EV	5.72	0.02

Note: Sample size (*N*) gives the total number of myrmecophile specimens per species used in network analysis. Note that additional specimens collected from army ant raids and refuse deposits were studied for species identifications (Table 1; Table S1). Network incidence (Inc.) indicates the number of times a myrmecophile was found in different host ant colonies. Diversity calculations are based on the bipartite army ant–myrmecophile interaction network (matrix provided in Table S1). Host diversity is given by the absolute number of host species (Hosts) and as effective Shannon diversity (e^{H}_{hosts}). Exclusiveness of host associations is given as standardized Kullback–Leibler distance (*d'*). Two species were denoted here as "false Lomechusini," which is a group of Neotropical rove beetles formally placed in the tribe Lomechusini (Elven et al., 2012). Asterisks (*) mark either species that have already been scientifically described as part of this project, or species where we have evidence via morphological inspection (including aedeagi dissections) that they represent species not yet scientifically described. Taxonomic species descriptions are in progress/planned for the following species: *Aphanister* sp. 1 (AKT), *Clientister* sp. 1 (AKT), *Euclasea* sp. 1 (AKT), *Sacosternum* aff. *lebbinorum* (M. Fikáček), the two *Ecitomorpha* species (MM), and all *Vatesus* species (CvB). The two *Ecitomorpha* species have erroneously been synonymized by Kistner under the species name *Ecitomorpha arachnoides* and need to be formally re-erected to species status (see von Beeren et al., 2018). In the two species complexes *Vatesus* cf. *clypeatus* and *Ecitophora* cf. *comes*, at least two of the three cryptic species are new to science. A careful taxonomic comparison to type material is necessary to evaluate this, which was beyond the scope of this study. A taxonomic revision of the subfamily Cephaloplectinae and Nossidiinae is currently in progress (WEH), including the taxonomically challenging ptiliid genera *Limulodes* and *Nossidium*. Abbreviations: EB = *E. burchellii*, ED = *E. dulcium*, EH = *E. hamatum*, EM = *E. mexicanum*, EL = *E. lucanoides*, EV = *E. vagans*.

important because interpretation of network metrics such as host specificity can otherwise be misleading (Ivens et al., 2016). We occasionally collected myrmecophiles for short periods of time at LSBS from colonies of other army ant genera: three *Neivamyrmex gibbatus* Borgmeier, 1953 colonies, one *Neivamyrmex pilosus* (Smith, 1858) colony, one *Neivamyrmex* cf. *asper* Borgmeier, 1955 colony, one *Neivamyrmex* cf. *impudens* (Mann, 1922) colony, one *Neivamyrmex* cf. *iridescens* Borgmeier, 1950 colony, and one *Nomamyrmex esenbeckii* (Westwood, 1842) colony. None of the associated guest species (17 species, 59 specimens barcoded; unpublished data) were found in *Eciton* army ants, except for the rove beetle *Tetradonia laticeps* Jacobson & Kistner, 1998, a known generalist myrmecophile

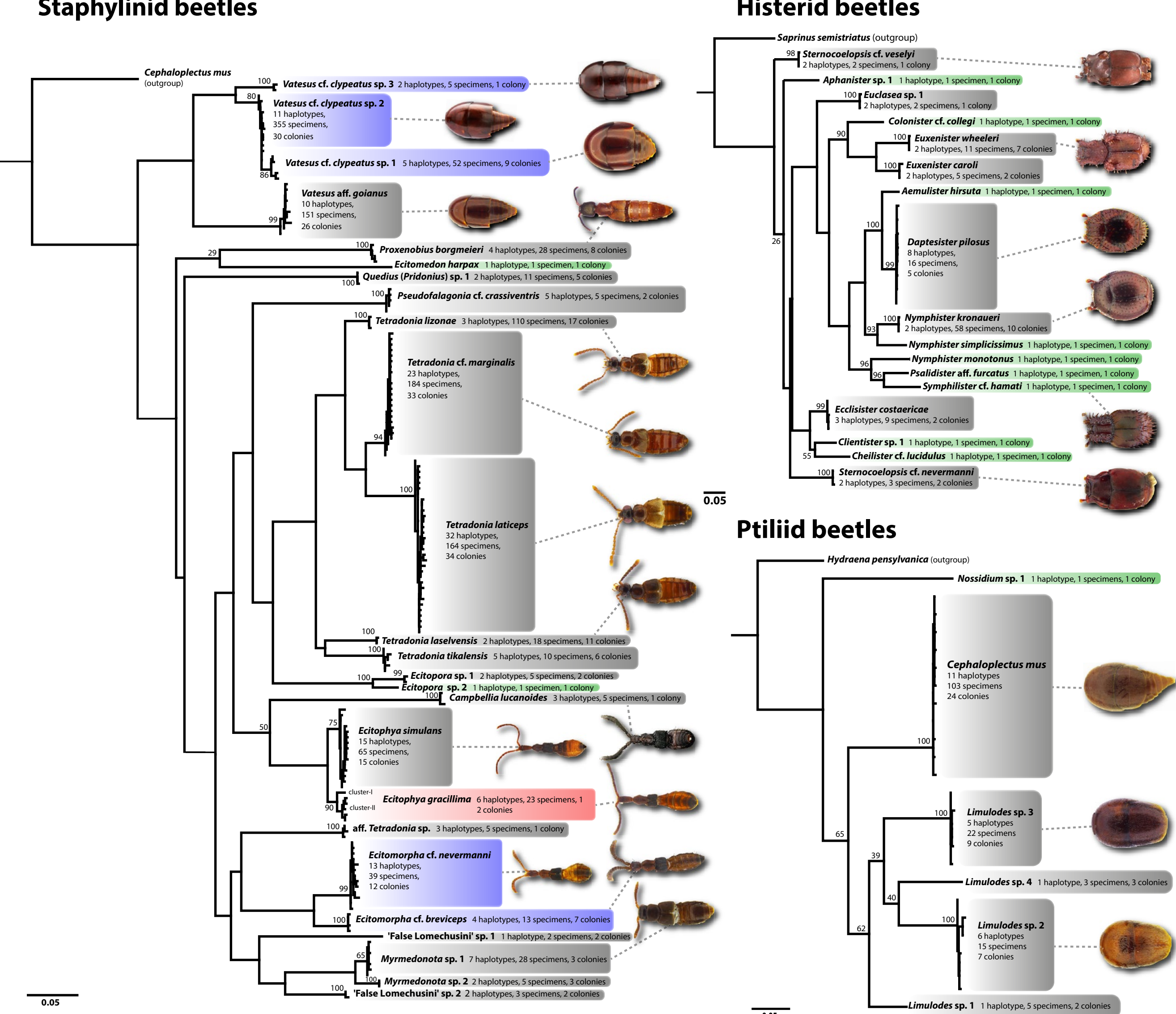


FIGURE 2 Molecular species identification in beetles. RAxML trees based on *COI* barcode data of staphylinid (rove) beetles, histerid (clown) beetles and ptiliid (feather-winged) beetles. Grey boxes show cases where morphological identification and DNA barcode clustering agreed on the presence of a single species. Green boxes depict singletons. Red and purple boxes highlight cases in which specimens initially identified as a single species split into two or more *COI* clusters. Additional morphological and genetic data suggested that those specimens either belonged to a single species (red boxes) or to different species (purple boxes; see also Supplementary Results). Scale bars show expected nucleotide substitutions per site as inferred by the RAxML algorithm. Bootstrap support values are shown at major nodes (1,000 repetitions)

of all six local *Eciton* species at LSBS (von Beeren et al., 2016b). This species was also found in one *Nomamyrmex esenbeckii* raid and one *Neivamyrmex gibbatus* emigration (Table S1).

3 | RESULTS

3.1 | Species identification and diversity of army ant myrmecophiles

We identified 2,355 myrmecophile specimens to the species level (Table S1). Of these, 61 specimens were identified based solely on morphological characters, while DNA barcodes were available for all other specimens as an additional character for identification (Table S1). Seventy-seven specimens could not be identified to the species level because DNA barcoding failed and because they belonged to taxa that we were not able to identify via morphology alone (54 phorid flies, 21 ptiliid beetles, two staphylinid beetles; Table S1).

Overall, we identified 62 myrmecophile species participating in *Eciton* army ant emigrations at LSBS: 25 rove beetles (Staphylinidae), 17 clown beetles (Histeridae), six featherwing beetles (Ptiliidae), one water scavenger beetle (Hydrophilidae), 11 scuttle flies (Phoridae), one millipede (Stylodesmidae) and one silverfish (Nicoletiidae) (Table 2). Among these, at least 14 species were new to science (Table 2). New species discoveries came in essentially two types:

cryptic species resolved by DNA barcoding, and new species that were morphologically clearly distinguishable but had not been collected or scientifically described.

We obtained 2,294 high-quality *COI* consensus sequences of army ant myrmecophiles (mean sequence length = 636 bp; range sequence lengths = 157–700 bp; Table S1). Amplification of *COI* repeatedly failed, or sequences showed low quality or stop codons in 138 specimens resulting in a 6% failure rate in DNA barcoding. BOLD's clustering algorithm RESL grouped 2,287 myrmecophile barcodes into 66 BINs, while no BIN was assigned to seven DNA barcodes due to short sequence lengths (Table S1). For 52 of these BINs, genetic clustering agreed with our morphological species identifications, meaning that morphologically distinguishable species were clustered in a single BIN. These BINs were also recovered in the RAxML tree analyses (Figures 2 and 3). Species identifications of the remaining cases (N = 14 BINs) are described and discussed in detail in the Supplementary Results and Figure S1.

In addition to the species presented in Figures 2 and 3, we analysed *COI* data from the following three species: the silverfish *Trichatelura manni* (Caudell, 1925) (13 haplotypes, 228 barcoded specimens, 57 *Eciton* colonies; maximum intraspecific p-distance: 0.95%; range of *COI* sequence lengths: 436—675 bp; BIN: BOLD:AEB1298), an undescribed hydrophilid beetle, *Sacosternum* aff. *lebbinorum* Fikáček & Short 2010 (five haplotypes, eight barcoded specimens, six *Eciton* colonies; maximum intraspecific p-distance: 1.37%; range of *COI* sequence lengths: 602—684 bp; BIN: BOLD:AEF2657), and the millipede *Calymmodesmus montanus* Loomis, 1964 (two haplotypes, three barcoded specimens, one *Eciton* colony; maximum intraspecific p-distance: 0.18%; range of *COI* sequence lengths: 544—592 bp; BIN: BOLD:AEH1114).

3.2 | Myrmecophile host specificity

Army ant myrmecophiles showed considerable variation in host specificity (host range: 1–6 host species; e^H range: 1.00–5.72; d' range: 0.00–0.52; N = 2,113 specimens; N = 70 *Eciton* colonies; Figure 4; Table 2). Four species infested colonies of all six *Eciton* host species in the community: the silverfish *Trichatelura manni* (e^H: 5.72; d': 0.02; N = 227 specimens; N = 57 *Eciton* colonies; Table 2), the featherwing beetle *Cephaloplectus mus* (e^H: 3.84; d': 0.10; N = 93 specimens; N = 23 *Eciton* colonies; Table 2), the phorid fly *Ecitophora* cf. *comes* sp. 1 (e^H: 5.51; d': 0.02; N = 105 specimens; N = 39 *Eciton* colonies; Table 2) and the rove beetle *Tetradonia laticeps* (e^H: 4.92; d': 0.08; N = 151 specimens; N = 28 *Eciton* colonies; Table 2). Most myrmecophile species were associated with a single host species (N = 39 species; N = 500 specimens; Figure 4; Table 2), resulting in a left-leaning host distribution curve (Figure S2). Many of these species were rare in host colony emigrations. Out of the 39 species occurring in a single host, 22 were only found in a single colony (N = 45 specimens), and of those, 16 were only represented by a single specimen (Table 2).

The following species were strict host specialists that were regularly found in host colony emigrations: the four ant-mimicking aleocharine rove beetle species *Ecitomorpha* cf. *nevermanni* (e^H: 1.0; d': 0.31; N = 18 specimens; N = 6 *E. burchellii* colonies; Table 2), *Ecitomorpha* cf. *breviceps* (e^H: 1.0; d': 0.31; N = 9 specimens; N = 6 *E. burchellii* colonies; Table 2), *Ecitophya simulans* (e^H: 1.0; d': 0.35; N = 46 specimens; N = 9 *E. burchellii* colonies; Table 2) and *Ecitophya gracillima* (e^H: 1.0; d': 0.38; N = 22 specimens; N = 11 *E. hamatum* colonies; Table 2); the staphylinine beetle *Proxenobius borgmeieri* (e^H: 1.0; d': 0.35; N = 29 specimens; N = 8 *E. hamatum* colonies; Table 2); the tachyporine *Vatesus*. cf. *clypeatus* sp. 1 (e^H: 1.0; d': 0.52; N = 57 specimens; N = 10 *E. vagans* colonies; Table 2); the histerid beetles *Nymphister kronaueri* (e^H: 1.0; d': 0.45; N = 58 specimens; N = 10 *E. mexicanum* colonies; Table 2) and *Euxenister wheeleri* (e^H: 1.0; d': 0.34; N = 16 specimens; N = 7 *E. hamatum* colonies; Table 2); and the two phorid fly species *Ecitophora bruchi* (e^H: 1.0; d': 0.46; N = 79 specimens; N = 11 *E. mexicanum* colonies; Table 2) and *Ecitophora varians* (e^H: 1.0; d': 0.52; N = 50 specimens; N = 10 *E. vagans* colonies; Table 2). Other species such as the rove beetle *Tetradonia lizonae* and the phorid fly *Ecitophora pilosula* had strong host preferences but infrequently occurred in other host species (Figure 4; Table 2; Table S1).

Interestingly, species of the two genera *Tetradonia* and *Ecitophora* showed a high intrageneric variability in host specificities. Host associations ranged from two to six species among the five *Tetradonia* rove beetles (Table 2; Table S1), with lowest host specificity in the species *T. laticeps* (e^H: 4.92, d': 0.08; N = 151 specimens from 28 *Eciton* colonies; Table 2) and highest host specificity in the species *T. lizonae* (e^H: 1.28, d': 0.35; N = 101 specimens collected from 14 *E. hamatum* colonies and one *E. burchellii* colony). In the seven *Ecitophora* phorid fly species, associations ranged from one to six host species (Table 2), with highest host specificity in *E. varians* (e^H: 1.00, d': 0.52; N = 50 specimens from 10 *E. vagans* colonies) and lowest in *E.* cf. *comes* sp. 1 (e^H: 5.51, d': 0.02; N = 105 specimens from 39 *Eciton* colonies; Table 2).

3.3 | Network-level specificity and network modularity

The associations between army ants and their guests differed from purely random associations (H_2' tested against 10,000 randomized networks: $p < .001$; incidences in the network matrix based on 2,113 specimens and 70 *Eciton* colonies; Table S1) and showed an overall moderate level of network specificity (H_2' = 0.47; Figure 4). This is also reflected in a moderate level of interaction specificity or exclusiveness of interactions of each army ant species (d': 0.38–0.53; Table 1), resulting from the fact that each *Eciton* species harboured myrmecophiles covering the entire range from host specialists to host generalists (Figure 4).

The army ant–myrmecophile network was significantly modular (Q tested against 10,000 randomized networks: $p < .001$) and showed a moderate degree of modularity (Q = 0.43; Figure S3). Each

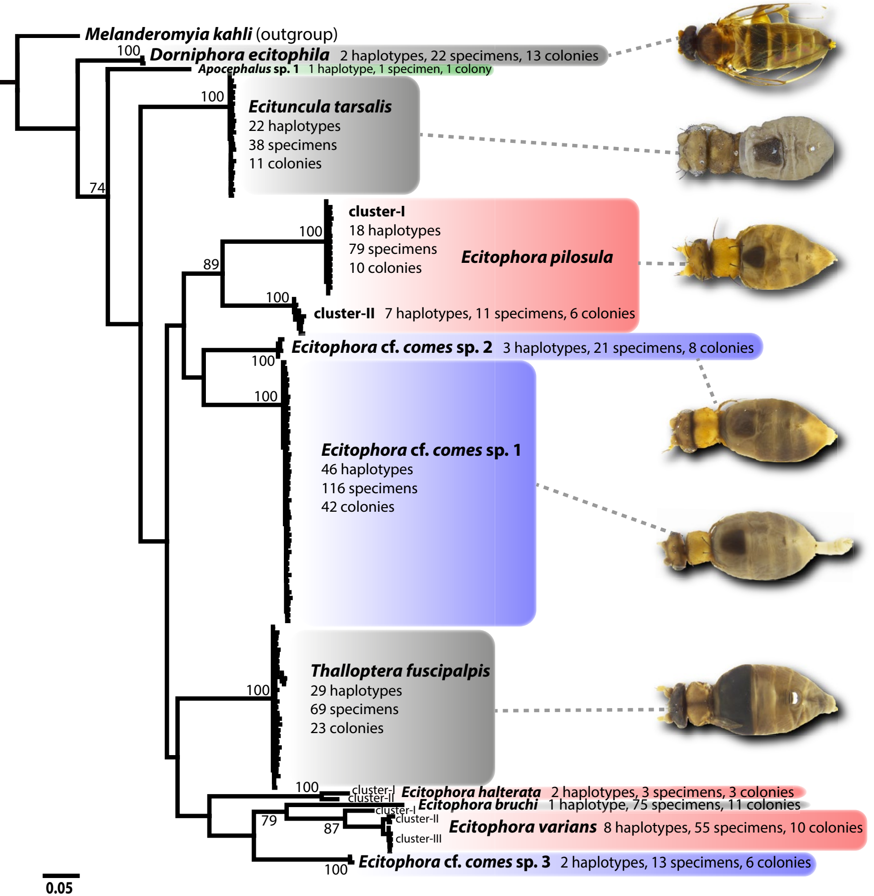


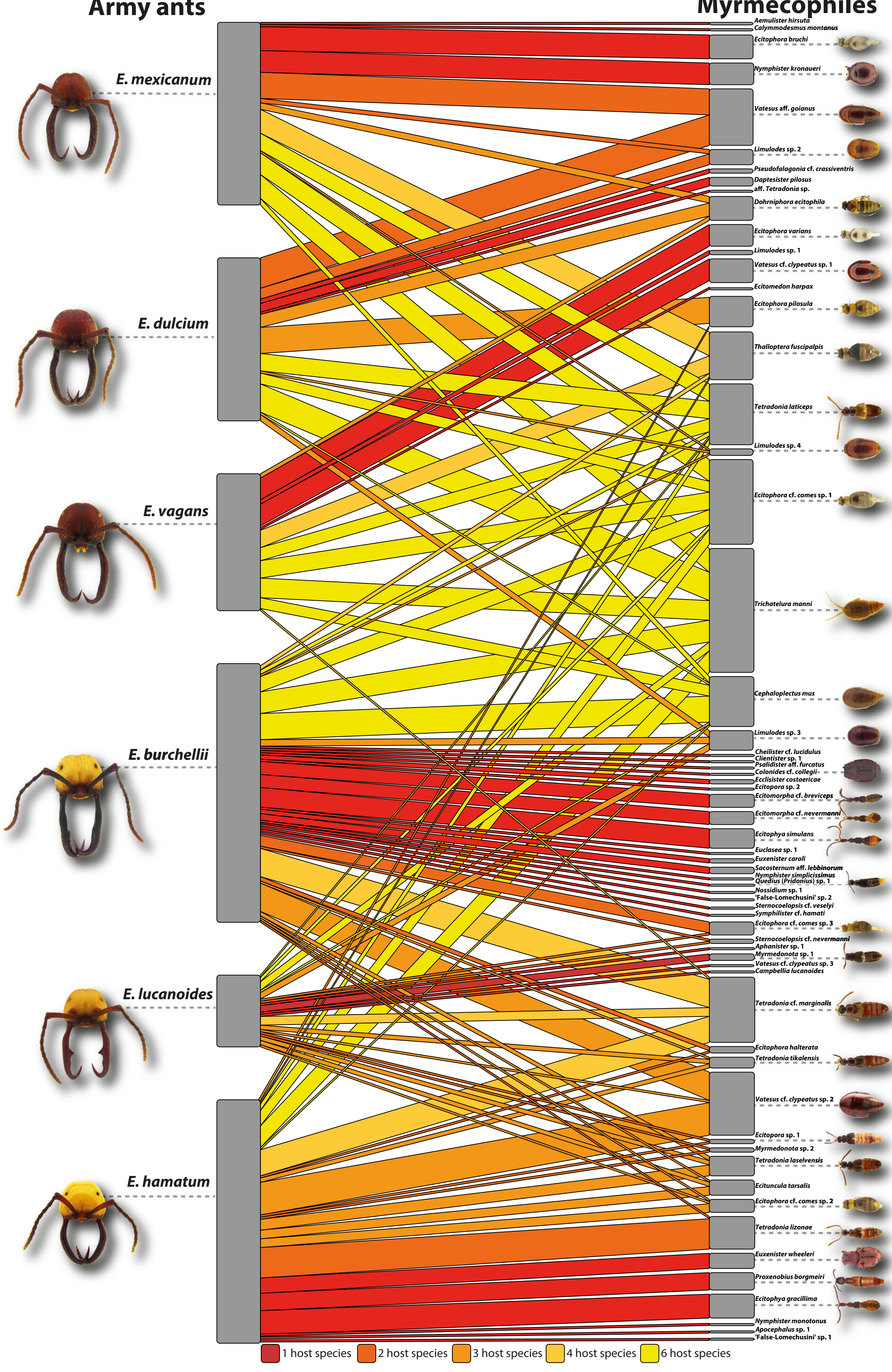
FIGURE 3 Molecular species identification in phorid flies. RAxML tree based on *COI* barcode data of phorid (hump-backed) flies. Grey boxes show cases where morphological identification and DNA barcode clustering agreed on the presence of a single species. The green box depicts a singleton. Red and purple boxes highlight cases in which specimens initially identified as a single species split into two or more *COI* clusters. Additional genetic data suggested that those specimens either belonged to a single species (red boxes) or to different species (purple boxes; see also Supplementary Results). Scale bars show expected nucleotide substitutions per site as inferred by the RAxML algorithm. Bootstrap support values are shown at major nodes (1,000 repetitions)

army ant species formed a single module within the network, except for the species pair *E. mexicanum* and *E. dulcium*, demonstrating a certain degree of host partitioning among myrmecophile species (Figure S3). We additionally tested for host partitioning by comparing network subsets consisting of two army ant species and all their associated myrmecophiles against null models of these network subsets. Each of the 15 possible network subsets differed significantly from randomized network models, showing that every *Eciton* species hosted a composition of myrmecophiles distinct from other *Eciton* species (all pairwise comparisons: $p \leq .018$; 10,000 permutations).

4 | DISCUSSION

The core of the present study was a biodiversity inventory of an army ant–symbiont community in a single population, which allowed us to describe the network of host–symbiont species interactions in unprecedented detail. Our survey unearthed numerous new army ant guest species. We have already described three of these species taxonomically (von Beeren et al., 2016b; von Beeren & Tishechkin, 2017), and we have erected one subspecies to the species level (Tishechkin et al., 2017). One of the new species is the charismatic histerid beetle *Nymphister kronaueri* with its exceptional mechanism of phoresy (von Beeren & Tishechkin, 2017) (Figure 1b). The discovery of many new species—in fact every fifth species in our survey was unknown to science (Table 2)—might at first seem surprising, in particular because LSBS is one of the best-studied tropical field sites and researchers have repeatedly collected army ant myrmecophiles there (e.g., Disney & Rettenmeyer, 2007; Jacobson & Kistner, 1998; Kistner & Jacobson, 1990; Kistner & Mooney, 2011; Tishechkin, 2007). However, we did expect to discover many new species due to the integration of DNA barcoding, a tool that facilitates the discovery of new species in taxonomically challenging groups (Hajibabaei et al., 2006; Hebert et al., 2004; Janzen et al., 2017; Pečnikar & Buzan, 2014). In army ant guests, this molecular technique had previously only been applied to very few selected taxa (Caterino & Tishechkin,

FIGURE 4 Bipartite interaction network between six *Eciton* army ant species and 62 myrmecophile species. In total, the network is based on 2,113 myrmecophile specimens collected from 70 *Eciton* colonies. Each species is represented by a grey box. Observed host–myrmecophile links are depicted by connecting lines, with line width proportional to the number of times a myrmecophile species was detected in different army ant colonies (i.e., the link strength). Network links are gradually coloured according to the number of host species in which they were detected, ranging from red (single host species) to bright yellow (six host species)


Army ants
Myrmecophiles
E. mexicanum
E. dulcium
E. vagans
E. burchellii
E. lucanoides
E. hamatum
Aemulister hirsuta
Calymmodesmus montanus
Ecitophora bruchi
Nymphister kronaueri
Vatesus aff. goianus
Limulodes sp. 2
Pseudofalagonia cf. crassiventris
Daptesister pilosus
aff. Tetradonia sp.
Dohrniphora ecitophila
Ecitophora varians
Limulodes sp. 1
Vatesus cf. clypeatus sp. 1
Ecitomedon harpax
Ecitophora pilosula
Thalloptera fuscipalpis
Tetradonia laticeps
Limulodes sp. 4
Ecitophora cf. comes sp. 1
Trichatelura manni
Cephaloplectus mus
Limulodes sp. 3
Cheilister cf. lucidulus
Clientister sp. 1
Psalidister aff. furcatus
Colonides cf. collegii
Ecclisister costaericae
Ecitopora sp. 2
Ecitomorpha cf. breviceps
Ecitomorpha cf. nevermanni
Ecitophya simulans
Euclasea sp. 1
Euxenister caroli
Sacosternum aff. lebbinorum
Nymphister simplicissimus
Quedius (Pridonius) sp. 1
Nossidium sp. 1
'False-Lomechusini' sp. 2
Sternocoelopsis cf. veselyi
Symphilister cf. hamati
Ecitophora cf. comes sp. 3
Sternocoelopsis cf. nevermanni
Aphanister sp. 1
Myrmedonota sp. 1
Vatesus cf. clypeatus sp. 3
Campbellia lucanoides
Tetradonia cf. marginalis
Ecitophora halterata
Tetradonia tikalensis
Vatesus cf. clypeatus sp. 2
Ecitopora sp. 1
Myrmedonota sp. 2
Tetradonia laselvensis
Ecituncula tarsalis
Ecitophora cf. comes sp. 2
Tetradonia lizonae
Euxenister wheeleri
Proxenobius borgmeiri
Ecitophya gracillima
Nymphister monotonus
Apocephalus sp. 1
'False-Lomechusini' sp. 1
1 host species
2 host species
3 host species
4 host species
6 host species

2006; Pérez-Espona et al., 2017). One example of cryptic species detected by DNA barcoding in our community survey were the three species included in the *Vatesus* cf. *clypeatus* complex (Figure 2). Each species had a high host specificity (Figure 4; Table 2). Identifications based exclusively on external morphological characters would have incorrectly identified these beetles as a single species with a relatively low host specificity at the species level (see also von Beeren et al., 2016a). Integrating DNA barcoding in future work on army ant guests, in particular at less explored field sites and on less studied, subterranean army ants, will certainly unearth plenty of additional new species. The herein published reference DNA barcodes for *Eciton* army ant guests can now serve as a backbone for such future work. Additionally, our study can also serve as a guide for future work of other host–symbiont interaction networks by portraying how systematic collection at the population level with species-level identifications and network analyses can be efficiently combined.

The integrative approach revealed considerable variation in host specificities among myrmecophile species. We found four species of host generalists—a silverfish, a ptiliid beetle, a phorid fly and a rove beetle—that associated with all *Eciton* host species in the community (Figure 4; Table 2). Note that host specificity is a relative term and the herein detected generalist species might still be relatively host-specific at the genus or tribal level when compared to myrmecophile species that infiltrate colonies of different ant subfamilies (e.g., Molero-Baltanás et al., 2017). The majority of myrmecophiles were, however, only detected from colonies of a single *Eciton* species (Figure 4; Table 2), resulting in a left-leaning host distribution curve (Figure S2)—a typical pattern of host–symbiont systems (Combes, 2005; Poulin & Keeney, 2008; Poulin et al., 2006; Schmid-Hempel, 2011). This pattern partly arose because many species were rare. For those species, including for example most histerid beetles, the actual host specificity at the population level remains elusive. In these cases, historical collections from other populations can sometimes help to assess the host specificity in more detail (e.g., von Beeren & Tishechkin, 2017; Kistner, 1979; Kistner & Jacobson, 1990; Rettenmeyer & Akre, 1966; Seevers & Dybas, 1943). For instance, we only collected four specimens of the histerid beetle *Euxenister caroli* in two *E. burchellii* colony emigrations (Table 2). However, additional collections from multiple Neotropical locations (>50 specimens from >11 *E. burchellii* colonies) corroborate that *E. burchellii* is indeed the species' preferred and possibly only host (Akre, 1968; Rettenmeyer, 1961).

Good examples of perfect host specialists—which we define here as myrmecophiles occurring regularly in host colonies of a single species—are the four ant-mimicking beetle species of the genera *Ecitomorpha* and *Ecitophya* (Figure 4; Table 2). Myrmecoid beetles show striking signs of specialization compared to their free-living relatives by mimicking host workers anatomically, behaviourally and chemically (Akre & Rettenmeyer, 1966; von Beeren et al., 2018; Maruyama et al., 2009; Maruyama & Parker, 2017; Parker, 2016). The evolution of such elaborate levels of specialization usually increases a symbiont's fitness on that particular host, but it comes at the cost of a reduced host range, which increases the risk of local coextinction events (Schmid-Hempel, 2011). As a consequence, specialists often coevolve and cospeciate with their primary host (Combes, 2005; Schmid-Hempel, 2011), which has recently been suggested for several species in the genera *Ecitomorpha* and *Ecitophya* (Pérez-Espona et al., 2017).

The high degree of host specificity in some of the studied myrmecophiles translated to strong signatures of host partitioning at the network level (Figure 4; Figure S3). A good example for host niche differentiation in closely related species are the four species of *Vatesus* beetles, which almost perfectly partitioned the available host niche space (Figure 4; Table 2). On the other hand, species in the rove beetle genus *Tetradonia* and the phorid fly genus *Ecitophora* showed substantial overlap in their host range (Figure 4; Table 2). This pluralism of host–myrmecophile interactions resulted in an overall moderate level of interaction specificity at the network level (H_2' =0.47; Figure 4). Because H_2' is a standardized metric, it allows for comparisons across communities and study systems (Blüthgen, 2010). For instance, more loose, nonsymbiotic mutualistic networks between ants and other organisms are generally characterized by less specific interactions (e.g., facultative ant–nectar plant associations: mean H_2' of eight networks = 0.23, H_2' range: 0.13–0.33; Blüthgen et al., 2007), while tight mutualistic symbioses with reciprocal partner dependencies usually show higher degrees of network specificities (e.g., obligate ant–plant symbioses: mean H_2' of 14 networks = 0.70, H_2' range: 0.23–1.00; Blüthgen et al., 2007).

Like mutualistic networks, parasitic ones can vary substantially in their level of specificity (e.g., H_2' =0.262 in frog-biting midges, Grafe et al., 2019; H_2' =0.77 in vertebrate ticks, Esser et al., 2016), often reflecting differences in the level of the parasites' host dependencies (e.g., Glasier et al., 2018; Poisot et al., 2013). Host dependency can be broadly divided into two categories: facultative associations where parasites opportunistically exploit hosts but can survive without them, and obligate associations where host exploitation is mandatory (Luong & Mathot, 2019). Both categories are represented among the manifold associations between ants and their guests (Brown & Feener, 1998; Glasier et al., 2018; Hölldobler & Wilson, 1990; Kistner, 1982; Molero-Baltanás et al., 2017; Parker, 2016). Among army ant associates, facultative associations exist in the detritivores and scavengers occupying the army ant middens, while myrmecophiles that have specifically adapted to follow the ants' emigrations are thought to form obligate associations (Gotwald Jr, 1995; Rettenmeyer et al., 2011). As we focused on emigration-following myrmecophiles, we expected most myrmecophiles would be obligate associates. However, at least some of the detected species might in fact form facultative associations, which seems particularly likely for several of the low-density myrmecophiles (see also Molero-Baltanás et al., 2017). For instance, we report here the first record of a millipede (*Calymmodesmus montanus*) in *Eciton* emigrations. These unusual guests are commonly found in army ant colonies of the genera *Nomamyrmex* and *Labidus* and are considered scavengers that do not depend on the ants for survival, but occasionally follow army ant emigrations to take advantage of the abundant food resources (Rettenmeyer, 1962).

Common ancestry is a frequent constraint that impacts host specificity in symbiotic organisms (Cooper et al., 2012; Esser et al., 2016; Mouillot et al., 2006; Park et al., 2018; Sasal et al., 1998). One example among the guests of *Eciton* army ants are the closely related myrmecoid beetles, all of which are associated with a single host species (von Beeren et al., 2018; Kistner & Jacobson, 1990; Maruyama & Parker, 2017). Conversely, there are numerous examples of symbionts without a phylogenetic signal in host specificity (Poulin et al., 2011; Poulin & Morand, 2014; Schmid-Hempel, 2011). In the studied community, species of two genera, *Tetradonia* rove beetles and *Ecitophora* phorid flies, showed high intrageneric variability in host specificities (Figure 4; Table 2). Apparently, phylogeny did not constrain the evolution of host specificities in these two genera and pre-adaptations allowed for both trajectories, the evolution towards host generalists and towards host specialists. As in the studied host–symbiont system, the underlying mechanisms leading to such variation in the degree of ecological specialization within a phylogenetic lineage remain unknown in the majority of cases, and understanding these mechanisms is still one of the most pressing issues in ecological and evolutionary parasitology (Poulin et al., 2011; Schluter, 2000; Thompson, 2005).

Adopting a phrase from E. O. Wilson, here we have unearthed "a remarkable legion of animal species" (Wilson, 1971, p. 389) that exploit the colonies of *Eciton* army ants in a Costa Rican community. Like so many of their cohabitants in tropical ecosystems, this legion is threatened by severe human interference with the natural world. The existence of many army ant associates depends on the presence of host ants (Gotwald Jr, 1995; Kronauer, 2020; Rettenmeyer, 1961). These, however, are sensitive to habitat degradation (Gotwald Jr, 1995; Kronauer, 2020) and local extinction of the ants will thus probably go hand in hand with an extinction cascade of numerous specialized, host-specific species, including many of the swarm-following birds and the diverse fauna of obligate myrmecophiles (Boswell et al., 1998; Brown & Feener, 1998; Koh et al., 2004; Kronauer, 2020; Kumar & O`Donnell, 2007; Pérez-Espona, 2021; Willis, 1974). Such coextinction cascades are indeed most severe in species forming tight symbiotic interactions, where at least one species depends entirely on the presence of one or a few others (Dunn et al., 2009). Here we have shown that many of the army ant-associated guests are highly host-specific and parasitize a single or a few host ants. These specialists certainly face high coextinction risks when host species disappear locally. Hence, we must enhance our efforts to protect tropical rainforests if we want to preserve army ants and their marvelous symbiont fauna.

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CONFLICTS OF INTERESTS

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

C.v.B. and D.J.C.K. designed and supervised the study. C.v.B., P.O.H., S.P., A.B. and D.J.C.K. performed field work. C.v.B. processed the collection. C.v.B., A.K.T., M.M., B.V.B., J.M.H. and W.E.H. identified species. C.v.B. acquired and analysed molecular data. C.v.B., N.B., A.B., and D.J.C.K. performed statistics. C.v.B. and D.J.C.K. wrote the manuscript, with feedback from all co-authors. All authors gave final approval for publication.

DATA AVAILABILITY STATEMENT

Sequences are deposited in GenBank and the Barcode of Life Data systems (for accession numbers see Table S1). Voucher specimens are deposited at 15 registered museum collections and the insect collection of the TU Darmstadt currently curated by C.v.B. (Table S1). Specimen images are deposited at BOLD systems (see Table S1).

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