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Unexpected bird–feather mite associations revealed by DNA metabarcoding uncovers a dynamic ecoevolutionary scenario

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Abstract

The high relevance of host-switching for the diversification of highly host-specific symbionts (i.e., those commonly inhabiting a single host species) demands a better understanding of host-switching dynamics at an ecological scale. Here, we used DNA metabarcoding to study feather mites on passerine birds in Spain, sequencing mtDNA (COI) for 25,540 individual mites (representing 64 species) from 1,130 birds (representing 71 species). Surprisingly, 1,228 (4.8%) mites from 84 (7.4%) birds were found on host species that were not the expected to be a host according to a recent bird–feather mite associations catalog. Unexpected associations were widespread across studied mite (40.6%) and bird (43.7%) species and showed smaller average infrapopulation sizes than typical associations. Unexpected mite species colonized hosts being distantly related to the set of their usual hosts, but with similar body size. The network of bird–mite associations was modular (i.e., some groups of bird and mite species tended to be more associated with each other than with the others), with 75.9% of the unexpected associations appearing within the module of the typical hosts of the mite species. Lastly, 68.4% of mite species found on unexpected hosts showed signatures of genetic differentiation, and we found evidence for reproduction or the potential for it in many of the unexpected associations. Results show host colonization as a common phenomenon even for these putatively highly host-specific symbionts. Thus, host-switching by feather mites, rather than a rare phenomenon, appears as a relatively frequent phenomenon shaped by ecological filters such as host morphology and is revealed as a fundamental component for a dynamic coevolutionary and codiversification scenario.

KEYWORDS
Acariformes, Analgoidea, Aves, coevolution, diversification, horizontal transmission, Sarcoptiformes

1 | INTRODUCTION

Speciation via host-switching (symbiont speciation after successful colonization of a new host species) is becoming acknowledged as one of the primary drivers for the adaptive radiation and diversification of symbionts (Bourguignon et al., 2018; Clayton, Bush, & Johnson, 2016; de Vienne et al., 2013; Nylin et al., 2017; Ricklefs, Fallon, & Bermingham, 2004). This conception, in contrast to strict

cospeciation, makes symbionts more active agents of their evolution. Mainly by cophylogenetic studies, we now know that the relevance of host-switching versus other processes such as cospeciation for the diversification of symbionts varies among groups (Clayton et al., 2016; de Vienne et al., 2013 and references therein). Evidence suggests that factors such as symbiont dispersal or parasite ecomorphology are related to speciation by host switch (Sweet, Bush, et al., 2018a; Sweet, Chesser, & Johnson, 2017), but we are still far from understanding both the genesis of these macroevolutionary patterns at an ecological and microevolutionary scale and which factors influence it.

The process of speciation by host-switching requires firstly some symbionts arriving to a new host (Clayton et al., 2016). These symbiont individuals are known as stragglers (Ròzsa 1993), a term which refers explicitly to individual symbionts that ended up on a different (new) host species. Stragglers rarely survive nor reproduce on the new host, but if they do, and if they eventually succeed spreading within the new host species, they are cataloged as host switches (Clayton et al., 2016; Rivera-Parra, Levin, Johnson, & Parker, 2017; Ròzsa 1993). This process may lead to genetic differentiation and finally to an event of host-switching speciation.

The existence of stragglers has been known for a long time, even long before the relevance of host-switching for symbionts speciation was revealed (Choudhury, Moore, & Marques, 2002; Horak et al., 2006; Kellogg, 1896; Ròzsa 1993; Shepherd & Edmonds, 1976). Rivera-Parra et al. (2017) provide a nice recent example. Studying feather lice from Galapagos Islands, they found stragglers in ca. 5% of the individual hosts examined while concluding that most stragglers would likely fail to colonize new hosts due to different ecological filters (see also Whiteman, Santiago-Alarcon, Johnson, & Parker, 2004). However, stragglers are still poorly documented in the literature as their observation is especially challenging because of their presumed scarcity and mostly ephemeral nature, especially for highly host-specific symbionts, which are unable to survive for long when not on or in their host species. In addition, according to these difficulties, stragglers are challenging to distinguish from methodological artefacts (e.g., sample contamination when collecting ectosymbionts from museum host specimens or from living hosts held together before sampling; Ròzsa 1993), thus likely being underrepresented in the literature.

Moreover, even for putatively highly host-specific symbionts such as feather mites on birds, they have been often described as multihost (or oligoxenous) symbionts (Dabert, Solarczyk, Badek, & Dabert, 2005; Doña, Proctor, Mironov, Serrano, & Jovani, 2018), and there are some evidence supporting that straggling and eventual host-switching to a new host may be a common phenomenon (Doña, Sweet, et al., 2017; Doña, Proctor, Mironov, et al., 2018; Gaud, 1992; Klimov, Mironov, & OConnor BM, 2017; Matthews et al., 2018). However, we are still far from quantifying the relevance of these processes and understanding the mechanisms governing them. In part, the study of stragglers and host switches at these scales has been hampered by the lack of appropriate methods to study this phenomenon. Here, we used DNA metabarcoding of feather mites to discover unexpected associations according to a recent comprehensive bird–feather mite associations catalog (Doña, Proctor, Mironov, Serrano, & Jovani, 2016) and study their ecological and genetic features to gain insight on host-switching dynamics at an ecological or microevolutionary scale.

Feather mites (Acariformes: Astigmata: Analgoidea and Pterolichoidea) are permanent and putatively highly host-specific ectosymbionts of birds (Dabert & Mironov, 1999; Dubinin, 1951; Gaud & Atyeo, 1996; Proctor, 2003; Proctor & Owens, 2000). Most species inhabit only one or a few, usually closely related, bird species (Doña, Proctor, Mironov, et al., 2018). Moreover, feather mites show specific adaptations to live on their hosts (Dabert & Mironov, 1999; Proctor, 2003): morphological fit to feather microstructure, microsite preferences within host feathers, fine-tuned distributions along entire bird wings, and behaviours to avoid feathers close to being moulted (Fernández-González, Pérez-Rodríguez, Hera, Proctor, & Pérez-Tris, 2015; Jovani & Serrano, 2001, 2004; Stefan et al., 2015). Feather mites lack specific life-history stages for transmission and except some members of the family Epidermoptidae and the genus *Strelkoviacarus* Dubinin, 1953 (Analgidae) are not known to disperse by phoresis on parasitic insects associated with birds, such as hippoboscid flies (Dabert & Mironov, 1999; Doña, Potti, et al., 2017; Jovani, Tella, Sol, & Ventura, 2001; Proctor, 2003). Current knowledge suggests that their primary mode of transmission is vertical from parents to offspring in the nest (Doña, Potti, et al., 2017). In addition, they likely maintain a mutualistic relationship with birds in which they feed upon fungi and bacteria and likely on the uropygial gland oil that birds smear on the plumage (Doña, Proctor, Serrano, et al., 2018). Thus, such host-specific symbionts have all the ingredients to be diversifying mainly by cospeciation. Interestingly, and contrary to this expectation, there is also evidence of horizontal transfer within and between bird species (Dubinin, 1951; Gaud, 1992; Jovani & Blanco, 2000), and recent studies have inferred that host-switching with subsequent speciation is the primary process driving their evolutionary diversification (Doña, Sweet, et al., 2017; Doña, Proctor, Mironov, et al., 2018; Klimov et al., 2017; Matthews et al., 2018). These results suggest that host-switching, despite its apparent difficulty for feather mites, has left macroevolutionary fingerprints along millions of years (Doña, Proctor, Mironov, et al., 2018; Doña, Sweet, et al., 2017). Our specific aims here were (a) to quantify the extent of unexpected associations in feather mites; (b) to study their performance (abundance) and genetic differentiation in the atypical hosts; and (c) to gain insight on the host–symbiont and mite infracommunity-level interactions that govern host-switching.

2 | MATERIALS AND METHODS

2.1 | Sampling and DNA metabarcoding pipeline

We sampled feather mites during 2010–2015 from live passerine birds captured with mist nets in different localities in Spain (Supporting information Table S1). We collected the feather mites found in primary, secondary and tertial feathers from the right wing

of each bird using a cotton swab impregnated with ethanol and preserved mites at −20°C in tubes with 96% ethanol.

We took particular attention to our sampling protocol to avoid the risk of artificial mite cross-contaminations between bird species (i.e., methodological artefacts rather than true unexpected mites). A previous study did not find feather mites detached from birds in cloth bags used to transport them from the mist net to the field station (Fernández-González, 2013). So, for 491 birds (of those from which we succeed sequencing their mites), we used "normal" field procedures. That is, we extracted birds from the mist net with bare hands, placed them in standard bird banding cloth bags and then handled them again with bare hands when sampling their mites using disposable cotton swabs (because of the obvious risk of cross-contamination by reusing them). Moreover, to test whether the prevalence found with this protocol came from cross-contamination when using bare hands or even reused cloth bags, we also applied a "refined" protocol to 639 birds where (a) we used single-use latex gloves for extracting each bird from the mist net. (b) A single-use paper envelope to carry the bird until the field workstation (some meters away) and store it till processing. (c) A new pair of disposable latex gloves for handling the bird during feather mite sampling using disposable cotton swabs. We found that the prevalence of unexpected mites did not differ between both protocols ("normal": 7.1% (35 out of 491) of samples with unexpected mites, versus 7.7% (49 out of 639) in "refined" samples; $\chi^2 = 0.04$; $df = 1$; $p = 0.8$). We also explored potential tagging errors (i.e., sticker tags which may have been mistakenly pasted to a different sample) by retrospectively checking whether natural hosts of stragglers were handled up to two birds before or after the focal bird with stragglers (i.e., birds potentially overlapping in time during sampling and thus susceptible of potential tagging interchanges). We found that in 70.5% of the cases, unexpected mites were unequivocally found even when a potential tagging error was highly unlikely (note that this does not mean that tagging errors are the cause of the remaining 29.5%). Overall, this shows that our field procedures were not introducing false-positive bird–mite associations, and therefore, we used samples from both protocols for downstream analyses.

Mites from each sample, representing a bird's mite infracommunity (i.e., each field microtube with feather mites from each bird), were counted under the stereomicroscope; that is, we counted the total number of feather mites from each individual bird, not the number of mites per mite species from each bird as identification of larvae, nymphs and some adult females by morphology is unfeasible. Then, we analysed each sample following the DNA metabarcoding pipeline for feather mites described in Vizcaíno et al. (2018). Briefly, each bird's mite infracommunity was placed into one well of a 96-well plate and filled with 96% ethanol, leaving two empty wells for a DNA-negative extraction control and a PCR-negative control. Then, DNA was isolated using the HotSHOT method (Truett et al., 2000). DNA-sequencing libraries were prepared by amplifying a region of the mitochondrial COI gene (Doña, Diaz-Real et al., 2015; Doña, Moreno-García, Criscione, Serrano, & Jovani, 2015; Doña, Proctor, Serrano, et al., 2018), and by adding the Illumina-specific sequencing primers, indices and adaptors in a two-step PCR. Finally, libraries were pooled together and analysed in a total of eight MiSeq 300PE runs (MISEQ REAGENT KIT V3). Wet-lab work was carried out at AllGenetics & Biology, SL (A Coruña, Spain) and sequencing at Macrogen (Seoul, Korea). Note that all libraries were pooled, that is, irrespectively of whether they were successfully amplified or not as most of the DNA quantifications were out of range for quantification but still potentially with enough DNA for high-throughput sequencing. Obtained reads were quality-checked and quality-trimmed. Specifically, the forward (R1) and reverse (R2) fastq reads of each MiSeq run were quality-checked with FASTQC (Andrews, 2010). And they were then imported into GENEIOUS v.8.1.7 (Kearse et al., 2012) for visual inspection and quality-trimming. We trimmed a region of variable length at the 3′ end of each file, according to the average Phred score (minimum quality score of 28) of each MiSeq run. The Python script (MMIS; Vizcaíno et al., 2018) was then used to automatize sequence concatenation, OTU picking and to eliminate mistagging events (i.e., a recently described sequencing artefact that results in 1% to 10% of reads misassigned to the wrong sample; Esling, Lejzerowicz, & Pawlowski, 2015; Sinha, Stanley, & Gulati, 2017; Owens, Todesco, Drummond, Yeaman, & Rieseberg, 2018). Moreover, only OTUs with more than 100 identical reads were kept. We also checked whether representative sequences contained stop codons. Overall, this sequencing-bioinformatic approach allowed us to get more than 300–400 bp of COI sequenced (after quality-trimming), a sequence length over the 200 bp minibarcode known to give similar results in species identification than the total length barcode (Doña, Diaz-Real et al., 2015).

We did not find any evidence of cross-contamination in our blanks when visualizing PCR gels (i.e., no band was visualized). Despite this, we sequenced each of the blanks. We did not find any read in 54 out of 55 blanks, and the one with reads contained a small number of reads (155) from *Pteronysoides parinus*. In particular, for this mite species, we only retrieved reads (16,746) in another sample from the same plate (i.e., among the 384 samples multiplexed in the same sequencing run). However, in that sample, this mite species was expected (i.e., these mites were collected from a *Cyanistes caeruleus* individual, a typical host for that mite species; Doña et al., 2016). On the other hand, these samples were distant wells (D1 vs. H12), suggesting that this not be a case of contamination due to pipetting. Thus, this case may be the single case in which we noticed our bioinformatic filter was not able to remove artefactual reads due to mistagging (see Vizcaíno et al., 2018 for more details). Mistagging is still relatively poorly understood (e.g., Costello et al., 2018), and further advances may be used to refine our analyses and identify our blank contamination as such. In any case, we are confident that this blank contamination do not compromise the validity of our study because the number of reads in the unexpected associations was much larger than the 155 reads retrieved in the contaminated blank (mean = 3,972; range = 106–33,102); 1 out of 55 (1.8%) potential contaminations is much lower than the 7.8% of unexpected associations we have found (see Results); and more importantly, S.M. a posteriori checked the actual presence of unexpected mites with a

morphological examination of the mites, finding them in 70.5% of the instances (see below).

2.2 | Data analyses

Unless otherwise stated, all analyses were carried out in the R environment (R Core Team, 2015). We labelled a bird–feather mite association as "unexpected" when this was not reported with confidence in the global catalog of bird–feather mite associations (i.e., data quality = 2 in Doña et al., 2016, hereafter "typical association"). This database reviews all available information on bird–feather mite associations from the literature, and S.M. taxonomically curated it carefully.

Samples containing representative sequences unclassified at the species level or containing unexpected mites were further analysed by S.M. based on morphological characters of the exoskeletons, thanks to the fact that our DNA extraction protocol preserves this material (Doña, Diaz-Real et al., 2015); that is, the same mite individuals were used for molecular and later on morphological analyses. In doing so, we registered the proportions of juveniles and adults for each infracommunity containing unexpected mites, although larvae and nymphs could not be assigned to any mite species. For adults, we also determined the numbers of males and females. Morphological identification revealed ten mite species which could not be associated with particular species through metabarcoding, and species from the *pinnatus* species complex were identified at the species level as they cannot be identified as different species using the COI region (Doña, Diaz-Real et al., 2015). Among these molecularly unidentified mites, we found five (a 7.2% of the total of mite species sampled in this study) putative new species belonging to five genera (*Alaudicola*, *Mesalgoides*, *Proctophyllodes*, *Scutulanyssus*, and *Trouessartia*) which were excluded for downstream analyses because of the impossibility of treating them as unexpected associations. Among the samples containing molecularly identified unexpected associations, 70.5% (43 out of 61) were also validated morphologically. From the 18 nonvalidated, eight (44%) contained nymphal stages in which species-level identification by morphology was not possible (Supporting information Table S2). In other words, the classical taxonomic analysis of the samples corroborated that the existence of unexpected bird–mite associations was not due to artefacts from the molecular analyses. Also, note that there were seven additional samples for which species-level identification according to morphological characters was inconclusive (Supporting information Table S2).

We estimated the intensity or infrapopulation size (i.e., number of individual mites) of each feather mite species found within each bird's mite infracommunity by multiplying the proportion of reads retrieved from each mite species by the total number of feather mites counted in each infracommunity and then rounding to the nearest integer. Differences in the number of primer mismatches in the primer annealing regions may potentially bias some of these estimations, especially in those species which were not studied in Vizcaíno et al. (2018). However, we have shown elsewhere that this yields a reasonable estimate of the number of individual mites (Diaz-Real, Diaz-Real et al., 2015; Vizcaíno et al., 2018).

For each feather mite species, we calculated when possible (see below) the genetic distances between unexpected mites and mites inhabiting typical hosts (according to Doña et al., 2016; hereafter nonunexpected mites) with the *dist.dna* function ("raw" model) from APE (Paradis, Claude, & Strimmer, 2004). First, we aligned representative DNA sequences from unexpected and nonunexpected mites of each mite species (only in this analysis we did not use sequences with stop codons) with MUSCLE v3.8.31 using default parameters (maximum number of iterations, 2) (Edgar, 2004). Then, alignments were trimmed to discard those columns which contained a significant proportion of gaps (i.e., to discard those sequences having different length consequence of having being sequenced and bioinformatically processed separately and potential low-frequency indels) using the function *msaTrim* with default parameters (fraction of gaps tolerated at the ends of the alignment, 0.5; fraction of gaps tolerated inside the alignment, 0.9) from MICROSEQ v1.2.2 (Snipen & Hovde Liland 2018). Also, we explored the distribution of haplotypes of unexpected mites by building haplotype networks with the *haplotype* and *haplonet* (using raw genetic distances) functions from PEGAS v0.10 (Paradis, 2010). Three species containing unexpected associations were identified only in the morphological assessments and therefore were not included in this analysis. For three other species, we obtained sequences from the unexpected associations but not from the typical hosts, so they were not included in this analysis either. Finally, *P. pinnatus* was also excluded from the genetic analysis as this a controversial taxonomic group whose species delimitation using COI is problematic (Doña, Diaz-Real et al., 2015).

Host phylogenetic information was obtained from BirdTree (Jetz, Thomas, Joy, Hartmann, & Mooers, 2012; http://birdtree.org). We downloaded 1,000 trees from the Ericson backbone tree and then summarized them by computing a single 50% majority-rule consensus tree using SUMTREE v 4.1.0 in DENDROPY v4.1.0 (Sukumaran & Holder, 2010, 2015), following Rubolini, Liker, Garamszegi, Møller, and Saino (2015). We found phylogenetic information for all the bird species we studied. Following Doña, Sweet, et al. (2017), Avibase information (accessed on March 2016; Lepage et al., 2014) was used to match avian taxonomy in Doña et al. (2016) with that of Jetz et al. (2012).

Following Doña, Proctor, Mironov, et al. (2018), we estimated the probability density function of the phylogenetic distances between host species sharing a mite species to study host phylogenetic specificity of unexpected and typical feather mites. To do so, we calculated the phylogenetic distance (as in Doña, Proctor, Mironov, et al., 2018) between each bird species pair sharing a mite species and calculated the proportion of bird pairs falling within ten phylogenetic distance bins (i.e., host range was split into 10 equal sized bins).

To understand whether host morphology imposes an ecological constraint to establishing onto a new host, we explored the relationships between the phylogenetic distance between typical and unexpected hosts and their differences in body size. Bird body mass is evolutionary conserved, so that closely related

species tend to have similar body sizes (Smith & Lyons, 2013). In addition, host body size is correlated with morphological variables of feathers that may constrain feather mite successful establishment, such as the interbarb distance of feathers (Pap et al., 2017). If unexpected hosts are phylogenetically distant from the typical hosts but with a similar body size, this would suggest that body size imposes a constraint to host-switching (Clayton et al., 2016; Smith & Lyons, 2013). For this purpose, we calculated the body mass differences and phylogenetic distances between all pairs of hosts in which a particular mite species was found (Doña et al., 2016). The phylogenetic distance was measured as the sum of branch lengths from the most recent common ancestor to the two tips (species) of the bird phylogenetic tree with the function *cophenetic.phylo* from APE v5.1 (Paradis et al., 2004). We measured body mass distance (i.e., the difference in the mean body mass for the two species) as the difference between the maximum (i.e., the heavier bird species) and the minimum (i.e., the lighter bird species) body mass of each pairwise comparison. We obtained body mass information from Dunning (2008). Body mass and phylogenetic differences were analysed using generalized linear mixed models (GLMM) (GLMER function from package LME4 1.1–12; Bates et al., 2015). We ran a Gaussian GLMM considering body mass distance as the response variable, the type of association (i.e., unexpected or typical) as the predictor variable, the phylogenetic distance as a fixed factor, and mite species as the random term. We confirmed assumptions underlying GLMMs by exploring regression residuals for normality against a Q-Qplot.

To further explore the ecology of the unexpected associations from a nonpairwise point of view, we first identified groups of birds and feather mites that tended to associate more among each other than with other species in the network of associations (i.e., modules), using the simulated annealing method implemented in the *netcarto* function with default parameters (iteration factor = 1; cooling factor = 0.995, bipartite = False) from RNETCARTO v0.2.4 (Doulcier & Stouffer, 2015; Guimera & Amaral, 2005a, 2005b). The adjacency matrix (i.e., a presence–absence matrix with hosts in columns and mites in rows) was built with all the bird–mite associations found in our DNA metabarcoding study (i.e., the unexpected and the typical). To evaluate whether hosts included in each module were more closely related than expected by chance (i.e., phylogenetic signal of hosts included in each module), we calculated the D-statistic using *phylo.d* function from CAPER v0.5.2 (Fritz & Purvis, 2010; Orme et al., 2013). The network was plotted using the *plotweb* function from BIPARTITE v2.08 (Dormann, Gruber, & Fruend, 2008).

3 | RESULTS

We collected mite infracommunities from 3,477 individual birds, from which we successfully built 3,090 libraries. Mainly because of DNA isolation failures, we eventually obtained sequences from 1,130 mite infracommunities (25,540 individual mites; 50 mite species identified by DNA metabarcoding, plus 14 mite species identified only by morphological characters; see Materials and methods) from 71 bird species.

We found unexpected mites in 84 bird individuals (1,228 individual mites; Supporting information Table S4), that is, 7.4% of the infracommunities and 4.8% of the individual mites studied. The presence of unexpected mites was not taxonomically restricted, but involved 43.7% (N = 31) of birds and 40.6% (N = 26) of mite species; 25.9% (14 out of 54) of the unexpected bird–mite associations were found in more than one bird individual (Table 1). Also, we found larval or nymphal stages in 30.9% (N = 22) of the mite infracommunities where unexpected mites were present and exoskeletons preserved for morphological analyses (N = 71), and in a 45.1% (N = 32), we found both males and females, thus suggesting reproduction (note that feather mites do not have dispersal stages) or potential for reproduction on that bird, respectively (Supporting information Table S2). The potential for reproduction may be even higher in those birds with females, as potential inseminated females alone have the potential for reproduction.

Excluding unexpected mites, most birds (94.6%; N = 1,017) bore one mite species, 5.2% (N = 52) had two, and only 0.2% (N = 3) had three mite species. However, in 70.2% (N = 59) of the birds with unexpected mites, these were the only mite species. In the remaining 29.8% (N = 25), unexpected mites shared the host with a typical mite species. Thus, unexpected mites were found coinhabiting a bird with another mite species more frequently than for typical mite species (i.e., 5.2% vs. 29.8%; χ^2 = 19.24; df 1; p < 0.001).

Overall, the average infrapopulation size (i.e., all the mites of a particular mite species occurring in an individual host) estimated for unexpected mites was smaller than that for typical species (Wilcoxon rank sum test; W = 43,042, p < 0.001). However, in some samples, some unexpected mites reached similar average intensities to typical mites (Figure 1). Among unexpected mites, mite infracommunities with reproductive stages (i.e., adult males and females; see above) showed higher intensity values (Wilcoxon rank sum test; W = 152.5, p < 0.001).

The minimum, mean and maximum genetic distances between sequences from unexpected and typical mite individuals showed different patterns among mite species. First, even maximum genetic distances between unexpected and typical mite individuals of the same species were lower than the mean smallest interspecific distances found for feather mites in Doña, Diaz-Real et al. (2015) in all cases (Figure 2). Second, in ten of the species inhabiting unexpected hosts, we found that at least some haplotypes found in unexpected mites were also found in the sequences of typical individuals (i.e., min distance = 0). However, in 68.4% (N = 13) of these mite species, mean or maximum genetic distances were above the median intraspecific distance of that mite species in typical hosts (Figure 2). Also, haplotype networks were overall more diverse in these species (i.e., with genetic distance values over the median) than in mite species with lower genetic distances, but differences in sample size may influence this (e.g., undersampled mite species may present artificial star-like structures; Figure 2; Supporting information Table S3). Lastly, for those mite species in which more than one typical host

TABLE 1 The number of mite infracommunities with unexpected mites found in each bird species. Numbers in parentheses indicate the total number of individuals sampled for that bird species

	Dolichodectes edwardsi	*Dolichodectes hispanicus*	*Joubertophyllodes modularis*	*Monojoubertia microphylla*	*Proctophyllodes cetti*	*Proctophyllodes ciae*	*Proctophyllodes clavatus*	*Proctophyllodes cotyledon*	*Proctophyllodes doleophyes*	*Proctophyllodes lusciniae*	*Proctophyllodes mesocaulus*	*Proctophyllodes motacillae*	*Proctophyllodes musicus*	*Proctophyllodes pinnatus*	*Proctophyllodes rubeculinus*	*Proctophyllodes schwerinensis*	*Proctophyllodes stylifer*	*Proctophyllodes sylviae*	*Pteronyssoides parinus*	*Scutulanyssus obscuroides*	*Trouessartia bifurcata*	*Trouessartia inexpectata*	*Trouessartia minuscula*	*Trouessartia reguli*	*Trouessartia serrana*	*Trouessartia trouessarti*
Acrocephalus arundinaceus (27)												1														
Acrocephalus melanopogon (16)					2							1														
Acrocephalus schoenobaenus (9)					2							1														
Acrocephalus scirpaceus (29)	2											1									1					
Carduelis carduelis (22)				2																						
Chloris chloris (7)				2													1									
Carduelis citrinella (2)														1		1										
Cettia cetti (39)							1			1				5			1		1							
Emberiza cirlus (8)				6		2																				
Erithacus rubecula (95)									1													1				
Estrilda troglodytes (2)									1														1			
Ficedula hypoleuca (50)				2																						
Galerida cristata (3)		1																								
Hirundo daurica (1)				1																						
Hirundo rupestris (1)																				1						
Hirundo rustica (50)												1														
Lanius excubitor (2)													1		1											
Locustella luscinioides (12)					8																					
Luscinia megarhynchos (67)														1												1
Luscinia svecica (46)												1					1									
Muscicapa striata (22)				1																						
Oenanthe hispanica (2)											2															
Oenanthe leucura (1)											1															
Oenanthe oenanthe (7)					1											1										
Phylloscopus bonelli (3)									1																	
Phylloscopus collybita (17)								1							1			1				3				
Phylloscopus trochilus (34)												1			1											
Regulus ignicapilla (2)																								1		
Saxicola torquatus (2)								1																		
Sylvia melanocephala (26)			1												4											
Turdus merula (19)																		1							2	

was sampled, genetic distances of unexpected infrapopulations of most mite species (seven out of eight; 87.5%) were in the range of variation of those of typical mites (Figure 2).

Unexpected mites inhabited hosts that were more distantly related than expected according to the relatedness of usual hosts of feather mite species in this study (Figure 3; Wilcoxon rank sum test; W = 476,650; $p < 0.001$). The same result was found for the global database of bird–feather mite associations (Figure 3; Wilcoxon rank sum test; $W = 101{,}250$; $p < 0.001$, Doña et al., 2016). However, unexpected mites were found on hosts which differed slightly less in body mass than typical hosts, but this difference was negligible at large phylogenetic distances (Figure 4; GLMM, unexpected vs. typical: $\chi^2 = 15.9$, $p < 0.0001$; phylogenetic distance: $\chi^2 = 40.0$, $p < 0.0001$; unexpected vs. typical × phylogenetic distance: $\chi^2 = 11.6$, $p = 0.0007$).

The bird–feather mite network was composed of 26 modules (Figure 5), with an average (min-max) of two mite species (1–8) and three (1–11) bird species per module. All modules but one were

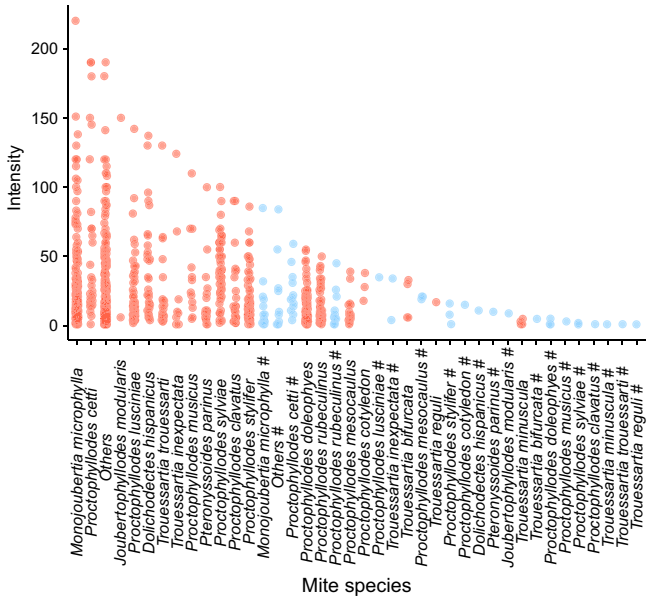

FIGURE 1 Scatter plot showing intensity values of feather mites' infrapopulations. Hash marks by species names and blued dots depict infrapopulations of unexpected mites while non-hashed names and red dots depict infrapopulations of nonunexpected. "Others" *x*-axis ticks group species only sampled either in typical or in unexpected associations

composed of hosts more closely related than expected by chance (mean (min, max) D = −1.19 (−4.4, −0.34); and (mean (min, max) Pr (D = 1) = 0.22 (0, 0.131; Supporting information Table S3). 75.9% of the unexpected bird–mite associations were found within the modules of the usual hosts of the same bird–mite association.

4 | DISCUSSION

Contrary to what was expected for these purportedly highly host-specific symbionts, we found a dynamic association scenario evidenced by a higher-than-expected frequency of unexpected associations (7.4% of the infracommunities and 4.8% of the individual mites). A rough calculation of unexpected feather mites in European passerines shows the relevance of our result. A conservative estimation of population size for European passerine species is of ca. 10^9 bird individuals (BirdLife International, 2017). This number, jointly with a conservative mean individual bird feather mite abundance of 10 mites per bird (Diaz-Real et al., 2014), leads to 10^{10} feather mites living in European passerines. Therefore, the prevalence of unexpected mites reported here yields a minimum of 10^8 individual birds with unexpected mites and 10^8 unexpected feather mites only for European passerines, which gives an idea of the potential relevance of unexpected associations for ecological and evolutionary processes.

Our results show that within these unexpected associations, there is a continuum of circumstances: mites recently "landed" on a new host species (stragglers) but with presumably low prospects of settling there; stable bird–mite associations that may have been overlooked in previous studies; and even long-lasting bird–mite associations that show enough genetic differentiation to suggest that they may eventually lead to an instance of host-switching speciation. Also, those unexpected mites found in closely related hosts may even be due to a process of codivergence or failure to speciate (i.e., host divergence without symbiont speciation; Johnson, Adams, Page, & Clayton, 2003). Cophylogenetic analyses using time-calibrated trees as well as population genomic analyses (e.g., Sweet et al., 2018) would probably shed light on these aspects, and further research integrating quantitative data (e.g., prevalence, intensity) is needed to understand the performance of the same mite species in different bird hosts.

Lack of bird–feather mites phylogenetic congruence (at low taxonomic ranks) and the power of host-switching to trigger further diversification have been shown elsewhere (Doña, Sweet, et al., 2017; Doña, Proctor, Mironov, et al., 2018; Matthews et al., 2018), and here, we provide evidence on how these patterns emerge from processes occurring at ecological and microevolutionary scales. Perhaps more importantly, a highly dynamic ecoevolutionary scenario where macroevolutionary patterns are only one of its outcomes is depicted, demanding to focus on the dynamics of these unexpected associations. In fact, we found a host–symbiont scenario in highly host-specific symbionts compatible with a geographic mosaic of coevolution in which network modules may be informative of the coevolutionary and codiversification dynamics (Thompson 1994, 2005; Poulin, 2010; Clayton et al., 2016; Ivens, Beeren, Blüthgen, & Kronauer, 2016; Pinheiro et al., 2016). The dynamics of the coevolutionary scenario of putatively highly host-specific symbionts, such as feather mites, could be analogous to that of a geographic mosaic of coevolution found in other systems in which populations are more connected (i.e., as the gene flow between symbiont populations inhabiting different hosts may be higher than previously thought), providing new avenues of research. In doing so, questions such as to what extent these dynamics are generalizable to other feather mite groups and what factors are in play should be addressed. For instance, feather mites of passerine birds may present a higher rate of straggling and host-switching than those of other bird groups (e.g., because potential donor–recipient hosts are morphologically more similar between them). Also, the dynamics of major host switches at this ecological scale could provide further valuable information (Doña, Proctor, Mironov, et al., 2018; Klimov et al., 2017). Moreover, habitat sharing may play an important role in straggling and host-switching dynamics and can vary through the year with different ecological processes (e.g., bird migration).

Also, our results provide important hints about the role of straggling and host-switching in the coevolutionary dynamics of bird feather mites. Interestingly, we found that unexpected associations reached, on average, lower infrapopulation sizes likely as a result of the lack of specialization on these hosts (Figure 1). Moreover, these associations were found in hosts from the same network module (which were composed by closely related birds). However,

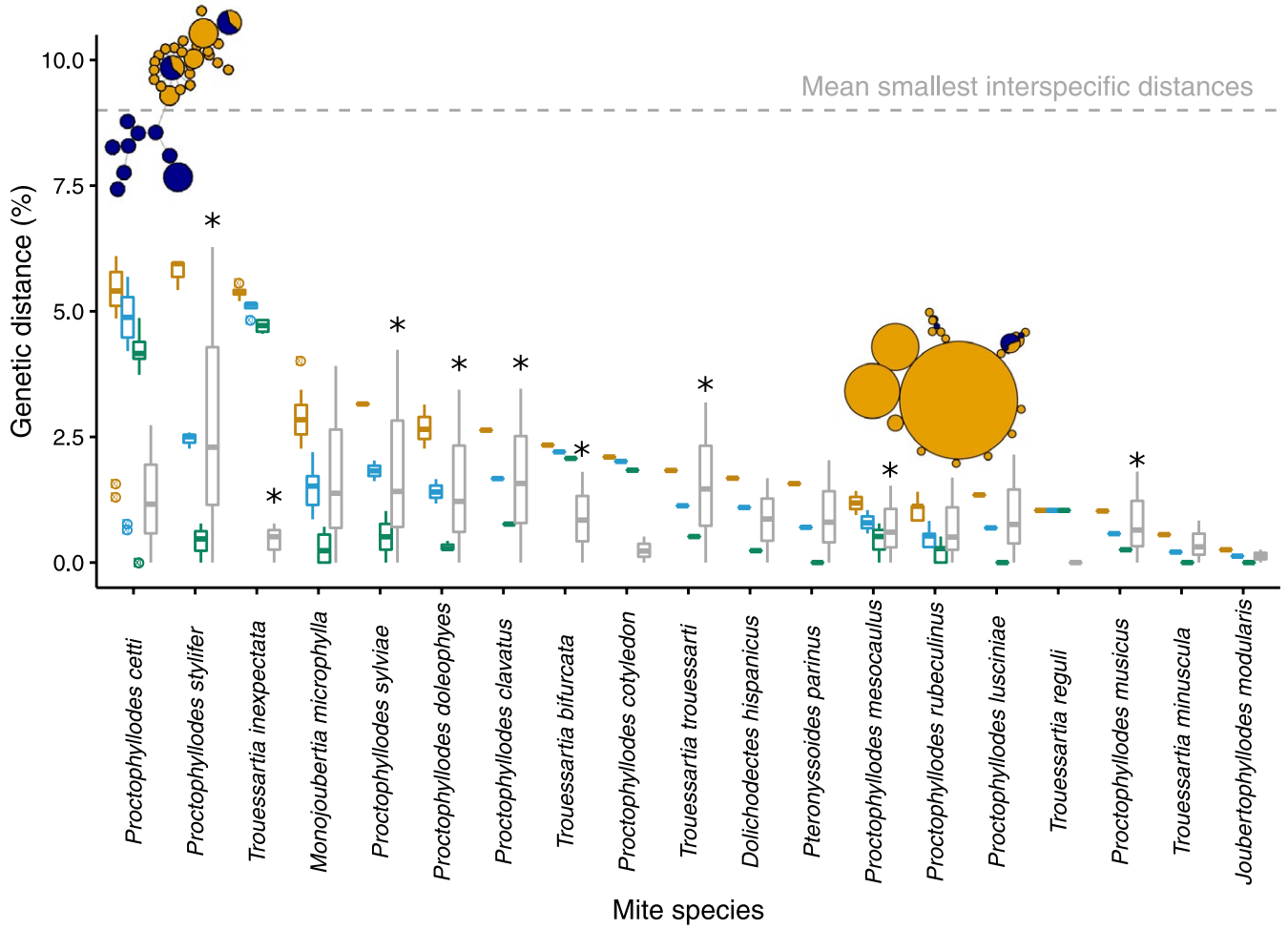

FIGURE 2 Boxplots showing the genetic distances of unexpected infrapopulations when compared to mites inhabiting typical hosts. Dashed grey line shows reference interspecific threshold for feather mites from Doña et al. (2015). Boxplots colours depict different statistics descriptors: orange (maximum), blue (mean), green (minimum) and grey, which depict intraspecific genetic distances for each mite species. Asterisks on the top of grey boxplots indicate mite species for which more than one typical (i.e., nonunexpected) host was sampled. Example haplotype networks showing contrasting diversity patterns belong to *Proctophyllodes cetti* (left) and *Proctophyllodes rubeculinus* (right). In yellow are depicted haplotypes of symbionts inhabiting typical hosts, and in blue, haplotypes of symbionts inhabiting unexpected hosts. Circle size is proportional to haplotype frequency

specifically, these hosts were more distantly related to the typical hosts than expected according to the phylogenetic host specificity of typical bird–feather mite associations (Figure 3). And this degree of relatedness was partially overlapping with the longest phylogenetic distances reported for typical associations in Doña, Proctor, Mironov, et al. (2018) (Figure 3). Finally, these unexpected associations were found in hosts with phylogenetic distances much shorter than potential associations with other bird species found in the same localities (e.g., mite species coming from non-passerine birds of the study localities would have introduced hosts in the analysis which would have shown phylogenetic distances above 100 in Figure 3). First, this supports that feather mites present a high phylogenetic host specificity (Doña, Proctor, Mironov, et al., 2018) not because of a lack of transmission opportunities, but likely because of strong ecological filters. Also, this shows that while most stragglers would probably not persist much time in their new hosts, some may succeed (and in fact, we have found mixed evidence of potential early-stage host-switching, and even of genetic differentiation). However, if they succeed, the comparison with typical associations strongly suggests that many of them would speciate due to host-switching, thus reducing the host range of the (parent) mite species again, although gene flow with the source host may persist and therefore influence the speciation process.

As already mentioned, our results advance in our understanding of the ecological filters encountered by mites once they reach a new host. The most plausible are those imposed by host morphology or other host traits with a strong phylogenetic signal, as evidenced by the short phylogenetic distances between hosts occupied by feather mite species in their natural host range. Feather mites may be only able to settle at least temporarily in those hosts that are morphologically similar to their typical hosts (Figure 3), so morphological traits related to body mass are potential candidates. Among them, wing flight feather traits such as interbarb distance would merit further study. Also, our results suggest that some of these filters may be not

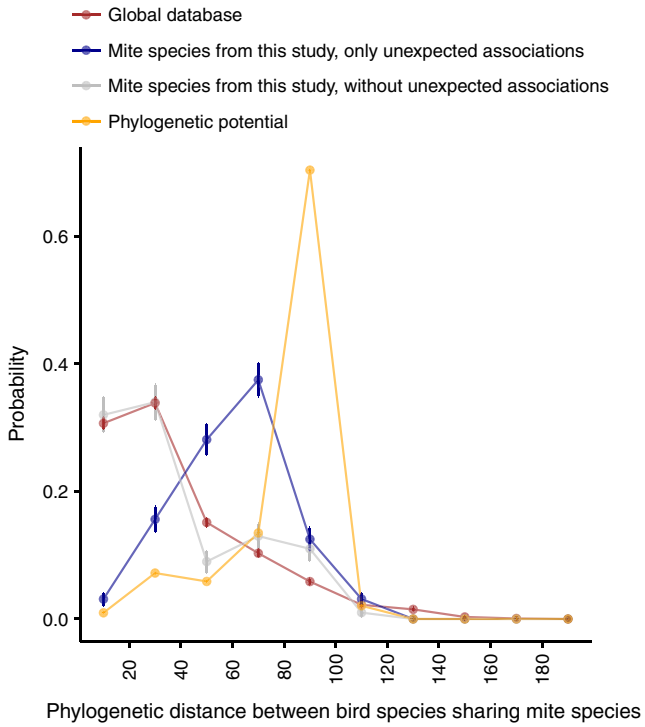

FIGURE 3 The probability that a pair of bird species sharing a feather mite species has a particular phylogenetic distance. Each line depicts probabilities of different mite subsets. Phylogenetic potential shows pairwise genetic distances between all hosts. Error bars represent confidence intervals (α = 0.05). Note that we only included mite species inhabiting more than one host

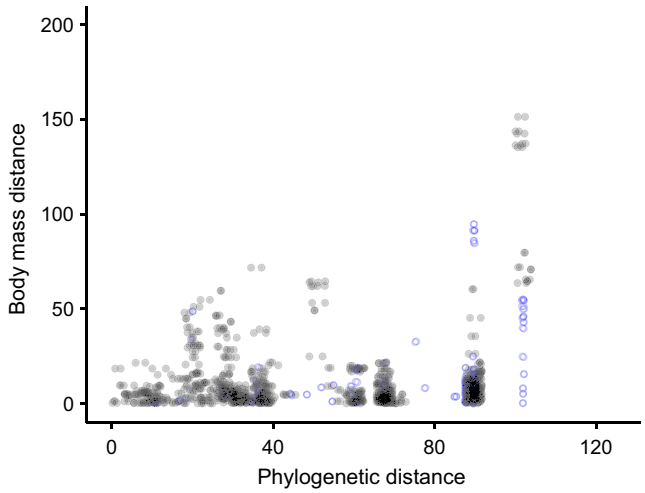

FIGURE 4 Scatter plot showing the differences in body mass between hosts sharing a mite species, accounting for the phylogenetic relatedness. Black circles depict pairwise comparisons between typical hosts, and blue circles depict comparisons including unexpected hosts. Points are horizontally jittered (two points) to improve visibility

related to host morphology. The fact that unexpected mites coexisted with another (typical) mite species in the same host infracommunity more frequently than typical mite species (which usually do not coexist with congeneric species in the same host infracommunity) suggests that interspecific competition may preclude host range expansion (Johnson et al., 2009; Fernández-González et al., 2015; Doña, Potti, et al., 2017). Indeed, feather mite species from the same genera rarely coinhabit the same host, likely as a consequence of interspecific competition (Doña et al., 2016). On the other hand, despite out of the scope of this study, *Trouessartia* and *Proctophyllodes* mite species have been found coinhabiting (but to some extent competing for space) the same hosts in higher prevalences than found here (Fernández-González et al., 2015). Our lower prevalence may be due to differences in prevalence among host populations (e.g., as found between sedentary and migratory blackcaps, Fernández-González et al., 2015) or due to detection problems intrinsic to our molecular approach. In this sense, Vizcaíno et al. (2018) showed that this methodology is prone to false negatives. So, while making our study conservative because among the false negatives there should be other unexpected bird–mite associations, it should be refined before using it for comprehensive catalogs of feather mite diversity.

Overall, these findings show that the host range of many of the studied feather mite species is larger than previously thought. This highlights the need of future studies aimed to understand mite transmission, with the potential of discovering unexpected ways of horizontal transfer between bird species. Most importantly, our study urgently encourages routinely integrating unexpected associations in host-symbiont studies and catalogs rather dropping them out as methodological contaminations and to study them as essential components to understand the link between the ecology and the macroevolution of host-symbiont systems.

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AUTHOR CONTRIBUTIONS

J.D., D.S., and R.J. conceived the study. J.D., D.S., and R.J. designed the study. J.D. collected the samples with the help of R.J. and D.S. J.D. analysed the data with the support of A.M., S.M, D.S. and R.J. S.M did the morphological identifications. J.D. wrote the manuscript with the help of all authors.

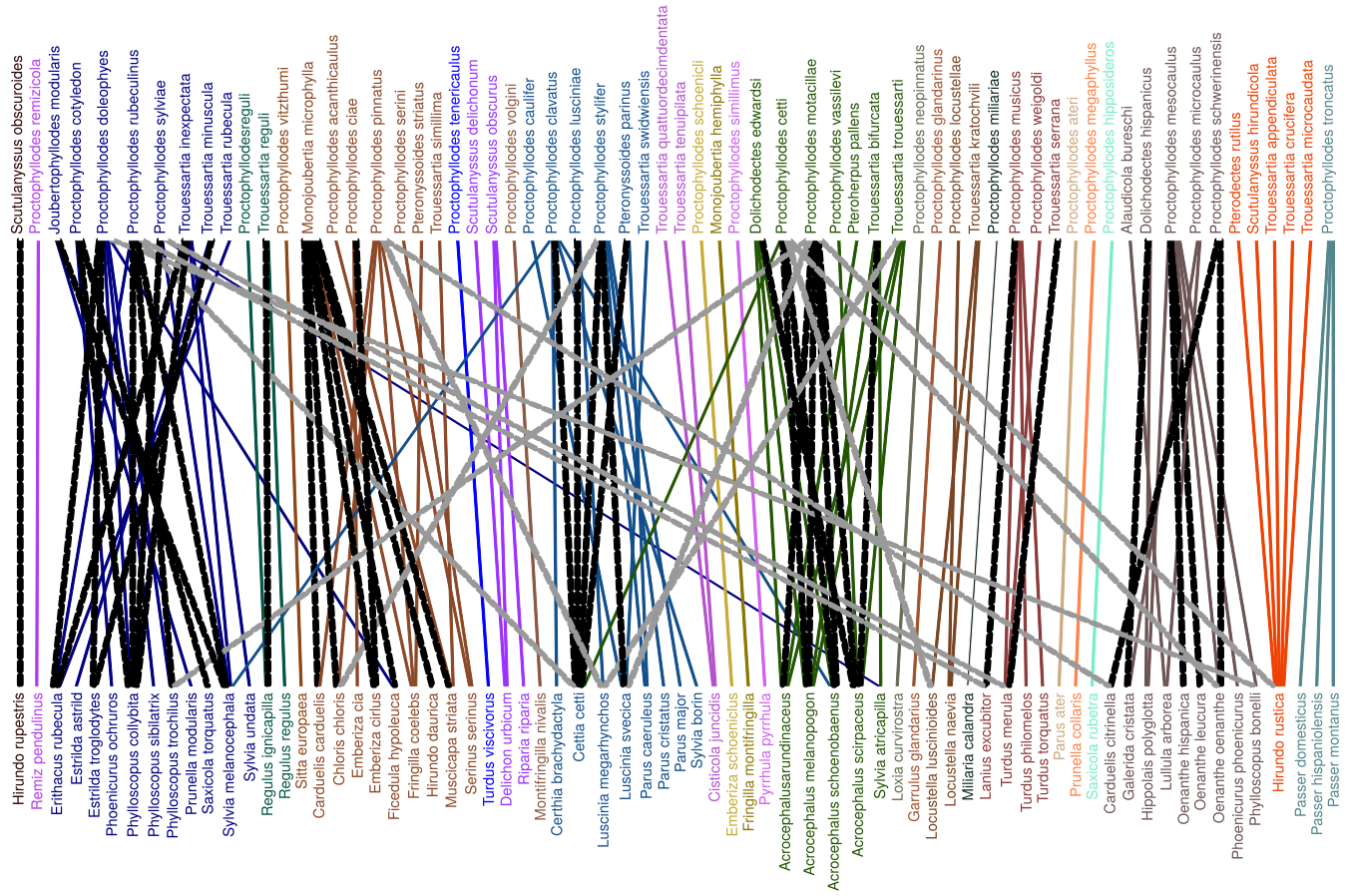

FIGURE 5 Feather mites and birds' ecological network. Colour labels depict module composition (mites above, host birds below). Link colours represent feather mite module composition. Thicker dashed black lines represent unexpected associations found in the same module, and thicker dashed grey lines represent unexpected associations found outside the module

DATA ACCESSIBILITY

We deposited HiSeq raw data and the processed representative sequences files in Figshare (https://doi.org/10.6084/m9.figshare.6384095; private link for review: https://figshare.com/s/f040ec6720733f7c742b).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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