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ORIGINAL ARTICLE

Mitogenomics and hidden-trait models reveal the role of phoresy and host shifts in the diversification of parasitoid blister beetles (Coleoptera: Meloidae)

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Abstract

Changes in life history traits are often considered speciation triggers and can have dramatic effects on the evolutionary history of a lineage. Here, we examine the consequences of changes in two life history traits, host-type and phoresy, in the hypermetamorphic blister beetles, Meloidae. Subfamilies Nemognathinae and Meloinae exhibit a complex life cycle involving multiple metamorphoses and parasitoidism. Most genera and tribes are bee-parasitoids, and include phoretic or nonphoretic species, while two tribes feed on grasshopper eggs. These different life strategies are coupled with striking differences in species richness among clades. We generated a mitogenomic phylogeny for Nemognathinae and Meloinae, confirming the monophyly of these two clades, and used the dated phylogeny to explore the association between diversification rates and changes in host specificity and phoresy, using state-dependent speciation and extinction (SSE) models that include the effect of hidden traits. To account for the low taxon sampling, we implemented a phylogenetic-taxonomic approach based on birth-death simulations, and used a Bayesian framework to integrate parameter and phylogenetic uncertainty. Results show that the ancestral hypermetamorphic Meloidae was a nonphoretic bee-parasitoid, and that transitions towards a phoretic bee-parasitoid and grasshopper parasitoidism occurred multiple times. Nonphoretic bee-parasitoid lineages exhibit significantly higher relative extinction and lower diversification rates than phoretic bee-and grasshopper-parasitoids, but no significant differences were found between the latter two strategies. This suggests that Orthopteran host shifts and phoresy contributed jointly to the evolutionary success of the parasitoid meloidae. We also demonstrate that SSE models can be used to identify hidden traits coevolving with the focal trait in driving a lineage's diversification dynamics.

Isabel Sanmartín and Mario García-París are Cosenior authors.

Isabel Sanmartín and Mario García-París contributed equally.

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KEYWORDS
diversification rates, evolutionary radiation, extinction rates, host specialization, parasitoids, state-dependent speciation

1 | INTRODUCTION

Speciation and extinction rates tend to vary over time and among clades as a response to changing conditions, including the origin of a new trait ("key innovation"), or an ecological opportunity resulting from the invasion of a novel niche (Barraclough et al., 1998; Donoghue & Edwards, 2014; Donoghue & Sanderson, 2015; FitzJohn et al., 2009; Freyman & Höhna, 2019; Losos & Miles, 2002; Maddison et al., 2007; Miller, 1949; Rabosky et al., 2013; Ricklefs, 2007; Sanderson & Donoghue, 1996; Stadler, 2011; Stanley, 1975). Changes in life history traits such as host switching or the development of new reproductive strategies are often considered powerful triggers of speciation bursts (Bonett & Chippindale, 2004; Hardy & Otto, 2014). Among these, host shifts can produce a major turnaround in the evolutionary fate of a parasitic lineage, and might result in a large increase in species number or in new levels of biological complexity (Erwin, 1992; Ricklefs & Fallon, 2002; Silva et al., 2012). Although host specialization is common in parasites (i.e., the "one-parasite-one host" rule), there is a growing body of literature showing that changes in host specificity are not infrequent (Braga et al., 2020; Hardy & Otto, 2014; Nylin et al., 2018). Through host jumping, parasites can escape extinction and increase their probability to persist over long evolutionary times (Thines, 2019) or shorter ecological time scales (Brooks et al., 2006; Calatayud et al., 2016).

Host shifts have been widely documented across organisms, for example, in humans, where the majority of pathogens originate through host changes, such as HIV, malaria, and the most recent SARS-CoV-2 (Wolfe et al., 2007; Zhang et al., 2020). Many factors intervene in the evolutionary success of a host shift, including physiological similarity between parasite and host (Runge & Thines, 2012; Thines, 2019); phylogenetic, ecological and geographical distance between current and potential hosts (Engelstädter & Fortuna, 2019; Göker et al., 2004); or differences in parasite and host mutation rates, that is, higher mutation rates may allow the parasite to overcome host defensive responses (Gandon & Michalakis, 2002). Thus, the likelihood of a novel host-parasite interaction and the evolutionary outcome of the new relationship are often not explained by a single character change (a "key innovation"), but by the interaction of multiple changes in different traits. On the other hand, host jumps that require changes in multiple levels of biological complexity (e.g., morphological, anatomical, physiological, or ecological) are difficult to reverse. Some authors consider host specialization as an evolutionary dead-end because the acquisition of a narrow set of food resources or hosts, and the concomitant trait adaptations, may limit opportunities for future diversification, in contrast to phenotypic plasticity in generalist species (Hardy & Otto, 2014).

Commonly known as "blister beetles", the family Meloidae (superfamily Tenebrionoidea) includes circa 3000 species (Bologna et al., 2008). Meloidae includes three subfamilies: Eleticinae, Nemognathinae, and Meloinae (Bologna, 2009; Bologna & Pinto, 2001; Pinto & Bologna, 1999). In the most recent phylogeny of the family (Bologna et al., 2008), Eleticinae was recovered as sister to the clade formed by Meloinae and Nemognathinae. Species in Eleticinae exhibit the nonparasitoid, free-living life cycle present in most Tenebrionoidea lineages (Bologna & Di Giulio, 2011; Pinto et al., 1996). In contrast, subfamilies Meloinae and Nemognathinae exhibit a unique, intricate "hypermetamorphic" life cycle, which involves three metamorphoses prior to the imago, and at least four larval phases.

Though the hypermetamorphic life cycle is present in all species of Nemognathinae and Meloinae, two traits exhibit variation at the tribal and generic levels: the mode of locomotion used by the first instar larva to reach the food source (Figure 1), and the host itself (Figure 2). All four tribes of Nemognathinae and six out of the eight tribes included in Meloinae are parasitoids of different species of solitary or subsocial bees (superfamily Apoidea), feeding on all resources available at the nest: eggs, bee larvae, and provisions (Figure 1). The two exceptions are Epicautini and Mylabrini: their first instar larvae feed on eggs from egg-pods of the family Acrididae, Orden Orthoptera (Figure 2; Bologna, 1991; but see Bologna & Di Giulio, 2011, for a possible case of parasitizing Sphecidae). So far, hypotheses on phylogenetic relationships among tribes and genera within Meloinae and Nemognathinae have been based on morphological characters (Bologna & Pinto, 2001; Denier, 1935; Kaszab, 1969; MacSwain, 1956; Selander, 1964), or combined data sets of morphological traits and DNA sequences from a few molecular markers (*16S* and *ITS2*, Bologna et al., 2008). In the latest study, (Bologna et al., 2008) proposed that Epicautini and Mylabrini were not sister-groups, and that host-specialization to grasshopper egg-pods occurred independently in the two tribes, representing a potential homoplasy in a “key innovation”.

In addition to differences in host type, the locomotion mode used by the first instar larvae of Meloinae to reach the food source can involve either active crawling or phoresy. In phoretic species, first instar larvae climb to flowers and latch to a passing bee to reach its nest (Figure 1; Hafernik & Saul-Gershenz, 2000); in nonphoretic species, larvae wander along the ground, actively searching for bee nests (Figure 1) or grasshoppers’ egg-pods (Figure 2). All Nemognathinae genera are phoretic bee-parasitoids (with the probable exception of *Stenodera*; Bologna et al., 2002; Bologna & Di Giulio, 2011), whereas some tribes of Meloinae that are bee-parasitoids (e.g., Meloini) include both phoretic (*Meloe*, *Lampromeloe*, *Eurymeloe*, etc,) and nonphoretic (*Physomeloe*) genera (Sánchez-Vialas et al., 2021). Bologna and collaborators (Bologna et al., 2008; Bologna & Pinto, 2001) suggested that phoresy evolved at least two times independently in the

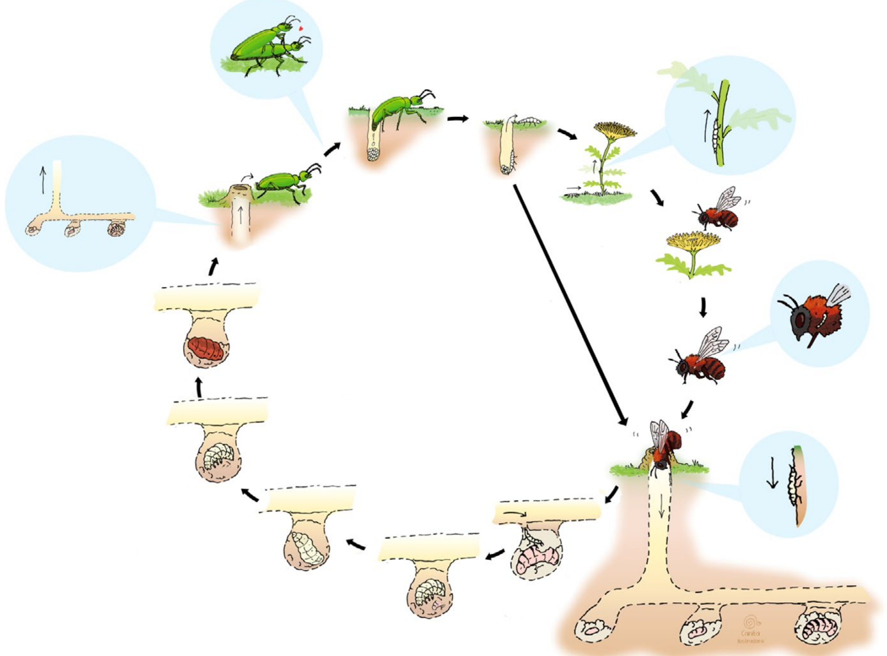


FIGURE 1 Life cycle of hypermetamorphic Meloidae parasitizing subsocial bees. Eggs are laid in the ground (Meloinae) or on the phyllaries of flowers (Nemognathinae) mainly Asteraceae (Enns, 1956). When the eggs hatch, a highly mobile larva emerges from the hatching site and searches for bee nests. This first larva ("triungulin") can either, depending on the lineage, climb a flower and wait for a bee to visit the flower and then attach to it and be transported to the nest (phoresy), or wander around the ground until it finds an entrance to the bees' nest (active searching). Once the first larva reaches the bees' nest, it starts eating the provisions, eggs or larvae of usually a single cell. A first metamorphosis then occurs, and the second larva known as "first grub larva" emerges; it presents reduced motility, but feeds nearly continuously until its metamorphosis. In the second metamorphosis, the first grub larva changes into a "coarctate larva"; the larva loses its appendages and enters into diapause. A third metamorphosis occurs, and a second grub larva morphologically similar to the second larva develops; this larva recovers the motility, although it does not feed. Finally, the larva pupates, and in a few days the adult emerges and the cycle begins again

bee-parasitoid Meloinae. These phylogenies, however, exhibited low statistical clade support, and were based on morphological traits directly associated to phoresy (Bologna et al., 2008).

Host specificity has been argued as the main factor to explain the remarkable difference in species richness between subfamilies Nemognathinae (c. 500 species) and Meloinae (~2500 species; Bologna et al., 2008). Host type might also explain differences in diversity levels among tribes of Meloinae: for example, Mylabrini and Epicautini, feeding on grasshopper eggs, include circa 600–700 species each, while the largest bee-parasitoid Meloinae tribes (Meloini and Pyrotini) do not exceed 300 species altogether (Table 1). There is, however, no obvious pattern of differences in species richness between the phoretic and nonphoretic genera of Meloinae (Table 1). For example, within tribe Lyttini, the nonphoretic genus *Lytta* comprises 109 species, while *Lagorina* includes only two. Some phoretic genera in tribe Meloini, such as *Meloe*, are species-rich (153 species), while others (*Spastonyx*, *Lyttomeloe*) comprise a few species (Table 1).

State-dependent speciation-extinction models (SSE) are a type of birth-death model that can be used to test for a statistical association between the heterogeneity in diversification rates observed within a clade and the rates of evolution of a focal character that is thought to be driving diversification, for example, a key innovation or ecological opportunity (Beaulieu & O'Meara, 2016; FitzJohn, 2010; Herrera-Alsina et al., 2019; Maddison et al., 2007). Recent studies have warned about the risk of overconfidence in SEE models, especially the issue of "pseudoreplication" and inflated Type I error (Maddison & FitzJohn, 2015; Rabosky & Goldberg, 2017). This issue has spurred the development of Hidden State-dependent speciation and extinction models (HiSSE, Beaulieu & O'Meara, 2016), allowing for the detected variation in diversification rates to be explained by unobserved ("hidden") traits, coevolving with the focal character. SSE models have been typically used in a maximum likelihood framework, but recent implementations employed Bayesian Inference to integrate the uncertainty in parameter estimates (Freyman & Höhna, 2019). A common handicap when using SSE models, or birth-death models in general, is incomplete taxon sampling (i.e., the reconstructed phylogeny does not contain all diversity in the clade of interest); this is even more severe in higher-level phylogenomic studies, where taxon sampling is often low. SSE models can account for incomplete taxon sampling by incorporating a parameter ρ that represents the global sampling fraction (Höhna, 2013). However, this ignores the fact that some clades are better represented than others in the phylogeny. Using clade-specific sampling fractions is one possible solution (Rabosky et al., 2014), but it may lead to incorrect estimations of extinction and diversification rates (Beaulieu, 2020; Moore et al., 2016).

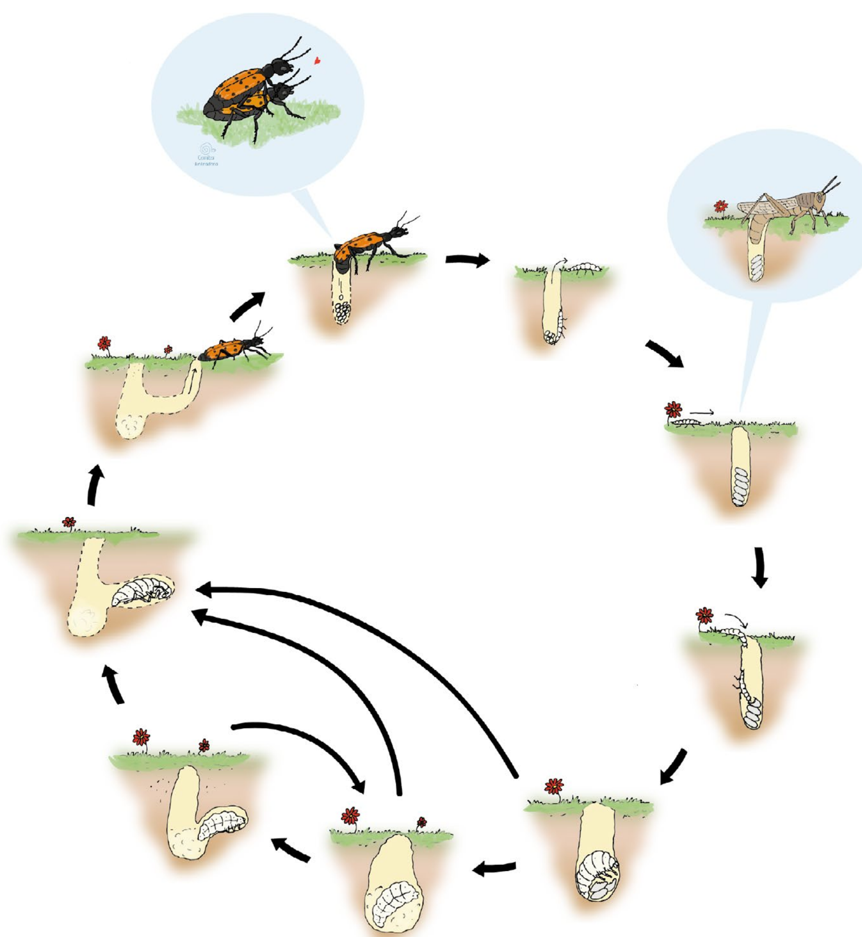


FIGURE 2 Life cycle of hypermetamorphic Meloidae specialized in grasshopper eggs. Meloid eggs are laid in the ground. A highly mobile larva emerges from the hatching site and actively searches for grasshoppers' egg-pods. Once the first larva reaches the pod, it starts eating the eggs. A first metamorphosis then occurs, and the second larva known as "first grub phase" emerges. The following larval phases are as those of the lineages that parasitize bees' nests. Jumps and reversions between larval stages have been observed mainly in Epicautini and Mylabrini (Selander & Mathieu, 1964; Selander & Weddle, 1969)

In this study, we reconstruct phylogenetic relationships and estimate lineage divergence times within the parasitoid subfamilies Nemognathinae and Meloinae, using whole-genome mitochondrial data and a representative taxonomic sample of Meloidae's closest relatives; we also generated a data set including additional nuclear markers, and samples from the free-living subfamily Eleticinae, in order to corroborate the mitogenomic phylogenetic relationships. This new mitogenomic phylogeny of the parasitoid blister beetles was used, together with SSE models, to estimate state-specific speciation and extinction rates. To account for the effect of incomplete taxon sampling in our estimates, we developed a methodological pipeline to infer diversification rates when there is low taxonomic sampling using a phylogenetic-taxonomic approach (Paradis, 2003). We also accounted for potential interactions with unobserved (hidden) traits, and incorporated uncertainty in phylogenetic and parameter estimation through the implementation of a Bayesian statistical framework.

Specifically, our aims were to: (i) Test previous phylogenetic hypotheses which were based on smaller data sets: Do the two parasitoid subfamilies of Meloidae, Nemognathinae and Meloinae, form a clade? Is there a sister-group relationship between the two tribes of Meloinae feeding on grasshopper's eggs, Mylabrini and Epicautini? Are phoretic clades independent lineages or constitute a monophyletic group? (ii) Test for a causal relationship between changes in diversification rates and state transitions in two traits regarded as key innovations: Are host jumping, phoretic behaviour, or both, driving diversification dynamics in hypermetamorphic meloids, and therefore responsible for the striking heterogeneity in species richness observed within this clade of blister beetles? (iii) Demonstrate how SSE models can be used for testing diversification dependence in relation to the trait of interest but also to identify unobserved, "hidden" traits, whose interaction with the focal character is responsible for shaping diversification dynamics within a lineage.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling for whole-genome sequencing

Our ingroup data set was composed of 29 mitogenomes of Meloidae: 23 species from 15 genera of Meloinae, representing six out of eight tribes in the subfamily: Epicautini, Eupomphini, Lyttini, Meloini, Mylabrini, and Pyrotini (Table 1), as well as six species from five genera of the sister subfamily Nemognathinae, representing two of the four recognized tribes: Nemognathini and Horiini (Table 1) (Figure 3). Of these 29 mitogenomes, 14 complete and seven partial mitogenomes were newly generated (Table S1), from specimens collected

TABLE 1 Taxa included in this study, with associated taxonomic diversity, presence of a phoretic behaviour, and host-type specialization. Species richness were obtained from Pinto and Bologna (1999), Bologna and Pinto (2002) and Campos-Soldini et al. (2018)

Family	Tribe	Tribe' species richness	Proportion of Sampled genera	Sampled genus	Genus' species richness	Phoresy	Host
Meloinae	Epicautini	564	1/5	*Epicauta*	492	no	grasshopper egg pods
	Meloini	160	4/6	*Lampromeloe*	2	yes	bee nest
				Eurymeloe	53	yes	
				Physomeloe	1	no	bee nest
				Spastonyx	2	yes	bee nest
	Lyttini	395	4/27	*Berberomeloe*	10	no	bee nest
				Lagorina	2	no	bee nest
				Lytta	109	no	bee nest
				Oenas	12	no	bee nest
	Eupomhini	26	2/8	*Megetra*	3	no	bee nest
				Tegrodera	3	no	bee nest
	Mylabrini	735	4/11	*Actenodia*	20	no	grasshopper egg pods
				Croscherichia	18	no	grasshopper egg pods
				Hycleus	450	no	grasshopper egg pods
				Mylabris	169	no	grasshopper egg pods
	Pyrotini	100	1/10	*Pyrota*	42	no	bee nest
Nemognathinae	Horiini	15	1/3	*Cissites*	2	yes	bee nest
	Nemognathini	500	4/24	*Apalus*	21	yes	bee nest
				Leptopalpus	2	yes	bee nest
				Zonitis	163	yes	bee nest
				Gnathium	14	yes	bee nest

in the field and stored in 100% ethanol at the Museo Nacional de Ciencias Naturales (MNCN-CSIC, Madrid, Spain). Mitogenomes of the remaining eight species were obtained from GenBank. We also obtained from GenBank ten additional mitogenomes from six families within superfamily Tenebrionoidea: Tenebrionidae, Mordellidae, Anthicidae, Oedemeridae, Mycetophagidae and Mycteridae (McKenna et al., 2015, 2019; Timmermans et al., 2015; Yuan et al., 2016), and which were used as outgroups. Table S1 gives a complete list of specimens and GenBank accession numbers. DNA extraction, sequencing and annotation methods are detailed in Supporting Information 1, and followed Uribe et al. (2017).

2.2 | Taxon sampling for Sanger sequencing of nuclear markers

The mitochondrial genome is typically regarded as a single, nonrecombinant locus of linked genes, and therefore no extensive gene tree discordance is expected among mitochondrial loci (Bernt et al., 2013). Yet, assuming that a single (mitochondrial) gene history represents the true organismal phylogeny can be misleading, especially if there is lineage sorting or reticulate evolution resulting from hybridization/introgression events (Maddison, 1997). We explored here this possibility by constructing a data set comprising partial sequences of two nuclear markers: *wingless* and *18S*. Specifically, we generated by Sanger sequencing DNA sequences for these loci from six and 18 Nemognathinae and Meloinae species, respectively, (covering the same tribal representation as in the mitogenomic data set). To test for the monophyly of the hypermetamorphic clade (Meloinae + Nemognathinae), we also downloaded sequences from GenBank of the 18S and wingless markers for two species of the nonparasitoid subfamily Eleticinae, *Iselma brunneipes* and *Iselma pallidipennis*. In addition, we downloaded a *16S* mitochondrial sequence of *Iselma brunneipes*, and extracted this same gene from our mitogenomic data set for all samples of Meloinae and Nemognathinae. This allowed us to build a three-marker nuclear-mitochondrial data set (MT+NC) that included representatives of all subfamilies within Meloidae, and which was based on evidence from the nuclear and mitochondrial genomes. We rooted this analysis using GenBank sequences of the selected nuclear and mitochondrial markers for one species of family

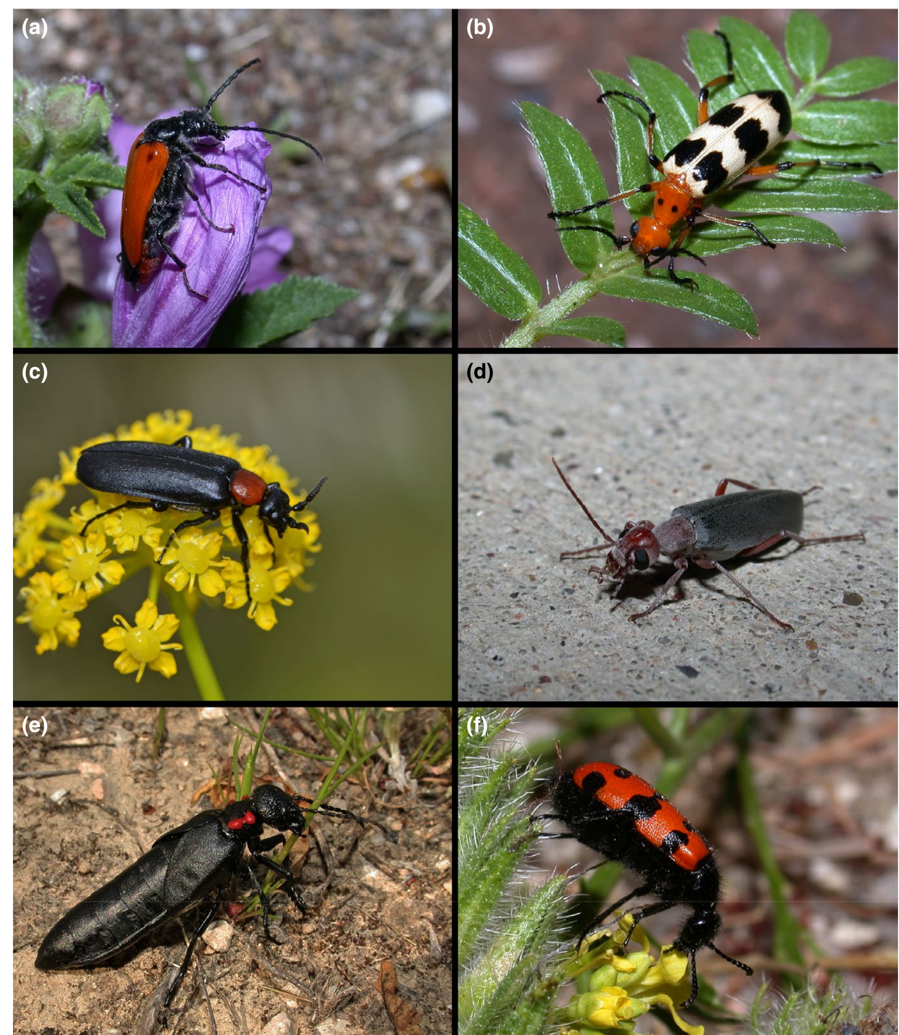


FIGURE 3 Habitus of representative species of Meloidae used for this study. Live adult specimens of: (a) *Apalus guerini* (Mulsant 1858), a phoretic bee specialist (from Perales de Tajuña, Spain) (Nemognathinae: Nemognathini); (b) *Pyrota palpalis* Champion 1893, a nonphoretic bee specialist (from Lordsburg, New Mexico) (Meloinae: Pyrotini); (c) *Oenas fusicornis* Abeille de Perrin 1880, a nonphoretic bee specialist (from Tielmes, Spain) (Meloinae: Lyttini); (d) *Epicauta tenella* (LeConte 1858), a grasshopper specialist (from Needles, California) (Meloinae: Epicautini); (e) *Physomeloe corallifer* (Germar 1818), a nonphoretic bee specialist (from Serranillos, Spain) (Meloinae: Meloini); (f) *Croscherichia paykulli* (Billberg 1813), a grasshopper specialist (from Moulay Bousselham, Morocco) (Meloinae: Mylabrini). Photographs by MGP

Anthicidae. A complete list of specimens included in this analysis and their GenBank accession numbers are provided in Table S2. DNA extraction, primers and PCR conditions for Sanger sequencing are detailed in the Supporting Information 1.

2.3 | Phylogenetic inference

Phylogenomic reconstruction was performed using only protein-coding and ribosomal RNA genes extracted from the complete mitogenomes (Abalde et al., 2017). DNA sequences of protein-coding genes were extracted, translated into amino acid sequences using TranslatorX Web Server (Abascal et al., 2010), and aligned using the MAFFT algorithm (Katoh et al., 2005). Amino acid and nucleotide raw alignments were trimmed with Gblocks (Castresana, 2000) using the following specifications: excluding many contiguous nonconserved positions and allowing gap positions within the final blocks. Ribosomal genes were aligned and cleaned through MAFFT and Gblocks online services (Katoh et al., 2017; Talavera & Castresana, 2007). We constructed two data sets: (a) NT-matrix, including all nucleotide sequences (12095 bp) and (b) AA+rNT-matrix, including DNA nucleotide sequences for the non-coding ribosomal genes and the translated amino acid sequences for the coding genes (5170 sites).

Maximum likelihood (ML) phylogenetic analyses were run on the NT and AA+rNT matrices, using the software RaxML v7.3.1 (Stamatakis, 2006). ML analyses were conducted with default parameters using the rapid hill-climbing algorithm and 1000 bootstrap pseudo-replicates. Bayesian inference (BI) analyses were performed using MrBayes v.3.2.6 (Ronquist et al., 2012). For BI, PartitionFinder v2 (Lanfear et al., 2016) was used to select the best partition scheme and molecular evolutionary models, under the Bayesian information criterion (BIC; Schwarz, 1978). We ran two chains of 100 million generations, sampling every 10000th.

Differences in biochemical profiles across sites can be an important source of systematic error in deep-time phylogenomics (Philippe et al., 2011). We used the site-heterogeneous CAT model implemented in the Bayesian program PhyloBayes (Lartillot et al., 2009) to analyse the NT and the AA (only) data matrices. We conducted analyses under the CAT-Poisson and the more complex CAT-GTR model, and including constant sites in the sequences, since excluding the latter can mislead phylogenomic inference (Thode et al., 2020).

Supporting Information 1 provides detailed descriptions of settings for these analyses.

To test if the grasshopper-parasitoid tribes Mylabrini and Epicautini, are independent lineages (Bologna et al., 2008), we used Bayes factors comparisons to evaluate two alternative hypotheses of relationships in terms of their support from the data: (H_0) Mylabrini and Epicautini are sister tribes within Meloinae; (H_1) Mylabrini and Epicautini do not form a monophyletic group. We used the stepping-stone sampling method (Xie et al., 2011) to estimate the marginal likelihood of each hypothesis/model, with 50 power posteriors as implemented in MrBayes. Supporting Information 1 provides more details on these analyses.

Finally, we constructed a nuclear-only matrix (18S+wingless), that together with the MT+NC matrix was used to test for the monophyly of the hypermetamorphic clade. To reduce the amount of missing data in the MT+NC-matrix, we merged the sequences from the two *Iselma* species. Phylogenetic analyses were ran in MrBayes on both matrixes, separately. We implemented a reversible jump MCMC strategy (command *lset nst = mixed*), which allows sampling across the substitution model space, without the need for prior model testing (Huelsenbeck et al., 2004). All other settings in the MCMC were set as in the mitogenomic analyses above.

Divergence times were estimated using Bayesian relaxed clocks implemented in BEAST v1.8.2 (Drummond & Rambaut, 2007), and the AA+rNT mitogenomic data set. Absolute times were obtained using calibration points based on fossil evidence, and independent molecular rates for the protein-coding and the ribosomal genes.

We did not use secondary age estimates from published molecular timetrees to calibrate the root node of our phylogeny because of the uncertainty regarding the age of family Meloidae (McKenna et al., 2015, 2019; Toussaint et al., 2017). The aforementioned studies provided fossil-calibrated timetrees for the entire order Coleoptera, including representatives of all major families. However, these studies differed in their use of fossil evidence and node priors, which resulted in differences in age estimates for the stem- and/or crown-node Meloidae (McKenna et al., 2019; Toussaint et al., 2017). Moreover, none of these studies included representatives of subfamily Nemognathinae. Because our interest here lies on the crown age of the hypermetamorphic clade of Meloidae, formed by Meloinae and Nemognathinae, we could not use these estimates; instead, calibration of the molecular rates relied on fossil evidence. A fossil specimen from the Dominican amber (Cordillera Septentrional, Dominican Republic) was identified by Poinar (2009) as a larva of *Meloe dominicanus*. This fossil was used to calibrate the crown-node of genus *Meloe* s.l. within Meloinae, comprising *Lampromeloe cavensis* and *Eurymeloe mediterraneus* in our data set. The age of the Dominican amber is controversial; in our analysis, we took a conservative approach, and constructed a lognormal prior distribution with a credibility interval spanning the oldest and youngest ages assigned to this site (50–15 Ma; Iturralde-Vincent & MacPhee, 1996; Cêpek in Schlee, 1990). See Supporting Information 1 for more details on the dating analyses and our calibration approach.

2.4 | State-dependent diversification

We employed State-dependent speciation and extinction (SSE) models (Beaulieu & O'Meara, 2016; FitzJohn, 2012; Maddison et al., 2007) to test for a causal correlation between the observed differences in species richness within the hypermetamorphic clade of Meloidae and variation in life-history traits such as host specificity and locomotion mode of first instar larvae. We implemented these SSE models within a hierarchical Bayesian MCMC framework using the open software RevBayes (Höhna et al., 2016); this allowed us to account for uncertainty in parameter estimation, that is, by computing marginal posterior probabilities for each parameter and ancestral state, as well as to use Bayes factor comparisons to assess the relative fit of SSE models to the data.

Incomplete taxon sampling has been shown to severely bias the estimation of diversification rates (Louca & Pennell, 2020; Morlon, 2014; Sanmartín & Meseguer, 2016; Stadler, 2009). Our mitogenomic phylogeny represents 1% of species diversity in the hypermetamorphic Meloidae (albeit tribal and generic diversity are represented up to 75 and 20%, respectively). RevBayes allows implementation of a global sampling fraction, representing the percentage over the total species richness, but this assumes random taxon sampling across clades. Clade-specific sampling fractions (Rabosky et al., 2014) are more adequate for our phylogeny, as some clades are better represented than others (e.g., *Epicauta* vs. *Pyrota*). However, such a procedure may lead to incorrect estimation of extinction rates in SSE models (see Supporting Information 1), and is not implemented in RevBayes.

Therefore, to account for the incomplete and uneven taxon sampling in our phylogeny, and to obtain a larger, clade-representative sampling to be analysed under RevBayes SSE models, we developed a methodological pipeline that relies on taxonomic information on species richness, the backbone phylogeny, the divergence times previously estimated in BEAST, and the use of birth-death simulations. Our approach mimics the phylogenetic-taxonomic method of Paradis (2003) to estimate diversification rates shifts in a higher-level phylogeny, and combines phylogenetic information—the timing of splitting events along the backbone of the phylogeny—with taxonomic information for each tip in the phylogeny (extant species-richness). In short, we simulated clade-specific diversity for several major clades (tribes and species-rich genera, Figure S1) in the empirical BEAST chronogram, using the mean age estimates and birth-death models to estimate a net diversification rate per clade; we then bounded the resulting simulated subtrees into the corresponding nodes in the backbone phylogeny to generate a supertree for the parasitoid Meloidae. To incorporate the uncertainty around the mean divergence times in the MCC tree, we repeated the approach over a subset of 100 time trees resampled from the BEAST 95% HPD posterior distribution; we then used the average value over all trees for the relative extinction rate and the net diversification rate per clade in all subsequent simulations. It is important to note that these simulated subtrees have no effect on the representation of character states in the phylogeny, as all simulated clades were

homogeneous for the trait in question, that is, each subtree has exactly the same state for all simulated tips. Supporting Information 1 provide a detailed rationale of our approach.

To test whether host jump or phoresy have been drivers of diversification in Meloinae, we implemented the binary state-dependent speciation-extinction (BiSSE) model (Maddison et al., 2007) in a Bayesian MCMC framework using the open software RevBayes. We coded the terminals for two states: parasitoids of bees (0) and grasshopper specialists (1) and in a separate analysis: (0) phoretic (1) and nonphoretic behavior. We used similar priors to Freyman and Höhna (2019) for the speciation and extinction rates and for the transition rates between character states; see Supporting Information 1 for more details. We also estimated two indirect parameters, the net diversification rate (speciation rate minus extinction rate) and the relative extinction rate (ratio of extinction to speciation). We set the global sampling fraction to 0.71 to account for incomplete taxon sampling in our supertree combining the empirical backbone phylogeny and the simulated clade-specific subtrees. The analysis was run with a chain length of 40,000 generations, sampling every 10th. We summarized ancestral states as nodal marginal posterior probabilities on the maximum a posteriori (MAP) tree, using code provided in the RevBayes website (https://revbayes.github.io/tutorials/morph/morph_more.html). Finally, we estimated the number and timing of transition events between states along the branches of the phylogeny, by employing a heuristic approximation to stochastic character mapping that does not require a rejection-sampling step (Freyman & Höhna, 2019). Stochastic character mapping was run in RevBayes with 500 time slices. Appendix S2-1 provides the script to run this analysis.

To explore the susceptibility of BiSSE to a type I error (Rabosky & Goldberg, 2015), we implemented the hidden state speciation extinction (HiSSE) model (Beaulieu & O'Meara, 2016). HiSSE includes two hidden states (A, B) within each of the two observed focal states ("host-type"); (analyses using phoretic behaviour as focal character did not show differences in speciation or extinction rates among states); in total, four character states: bee parasitoids (0) with hidden states A and B, resulting in the joint observed*hidden states 0A and 0B, and grasshopper specialists (1) with hidden states A and B (1A and 1B). If differences in speciation and extinction rates detected by BiSSE for the observed character states 0 and 1 are maintained in the two hidden states (A and B), we may conclude that heterogeneity in diversification rates across clades is explained at least partly by the focal character traits. When this is not the case, the observed differences in species richness cannot be associated to the focal character, but to a hidden trait to which diversification is related (Freyman & Höhna, 2019). We used similar prior and analysis settings as for the BiSSE analysis, except that transition rates between the hidden character states were set to be equal; see details in Supporting Information 1. Stochastic character mapping was also employed. Appendix S2-2 provides the full script to run this analysis.

The HiSSE analysis revealed a "hidden" trait which, along with host type, was associated to changes in diversification rates, and which exhibited a distribution of states across tips that corresponded to the trait "phoresy" (see Results). To test for the joint effect of phoresy and host-type on the diversification rates of the hypermetamorphic clade of Meloidae, we ran a third analysis using the multiple state-dependent speciation and extinction (MuSSE) model (FitzJohn, 2012). We considered three character states: (0) "phoretic parasitoids of bees": that is, lineages that feed on bee eggs, larvae and provisions, and that use adult bees as a mean of transport to reach the nest; (1) "nonphoretic parasitoids of bees", that is, lineages that feed on eggs, larvae and provisions of bees and use crawling on the ground to reach the nest; (2) "grasshopper parasitoids: lineages that feed on Acrididae eggs and use ground crawling to find the egg pod. Notice that phoresy only occurs in bee-parasitoid, that is, there are no Meloidae lineages that are "phoretic grasshopper" parasitoids. Therefore, we could not model the joint evolution of phoresy and host-jump as two independent binary traits. Settings for the MuSSE analysis followed those in BiSSE (see Supporting Information 1). However, due to the higher model complexity, a preburnin step of 5000 generations was needed for parameter auto-tuning before running a final MCMC chain length of 20000 generations. The script to run MuSSE is shown in Appendix S2-3.

To test the robustness of the MuSSE results to Type I error, we ran a multiple-state HiSSE analysis (MuHiSSE) with two hidden states (A, B) associated to each of the three observed states (0, 1, 2). In all, our MuHiSSE model included six states (0A, 1A, 2A, 0B, 1B, 2B), and used the same priors as the HiSSE model. Appendix S2-4 provides the script for this analysis.

Though HiSSE-type models above can account for the effect of hidden traits driving diversification shifts, it still assumes that heterogeneity in diversification rates across clades is linked to covariation with a trait evolving along the phylogeny. It might be the case that other mechanisms explain clade differences in species richness, for example, in response to abiotic factors (Rabosky & Goldberg, 2017). To provide a null model to compare against the MuSSE model, allowing clade heterogeneity but without assuming trait-dependent diversification, we implemented a character-independent diversification (CID) model (Caetano et al., 2018) in RevBayes. We implemented a CID2 model with two hidden states (A and B) within each of the observed character traits (0, 1 and 2). Contrary to HiSSE, speciation and extinction rates for the focal states were constrained to be equal, but were allowed to vary among the hidden states, that is, 0A = 0B, 1A = 1B, 2A = 2B. Thus, rate-heterogeneity is included in the CID2 model but unlinked to the focal trait (Caetano et al., 2018). The model also includes six transition rates for the joint observed*hidden states. Prior settings followed those in MuSSE. The script to run this analysis is provided in Appendix S2–5.

Figures S2–S6 represent each of the SSE models described above as directed acyclical graphs (DAGs), indicating parameter dependencies and prior distributions; DAGs were plotted with the graphical software GraphViz (Ellson et al., 2004). We estimated the support for each SSE model using Bayes Factor comparisons among the marginal likelihoods; these were estimated via path sampling and stepping-stone sampling analyses in RevBayes. The script to run this analysis is provided in Appendix S2-5.

Finally, we examined the robustness of our SSE results against phylogenetic uncertainty in our simulated approach, that is, we tested for the effect of selecting a particular subtree, among the 100 simulated, to represent clade diversity for a given node in the empirical-simulated phylogeny. First, using an R loop script (Appendix S2-6), we generated 100 empirical-simulated phylogenies, with alternative random subtrees selected and bounded for clades in the backbone phylogeny. Second, we checked that all simulated phylogenies belonged to the same "congruence diversification class" (Louca & Pennell, 2020). Third, we ran a MuSSE analysis, with the same settings as above, on each of these 100 empirical-simulated phylogenies, and then compared posterior estimates of state-dependent speciation and extinction rates among them. The aim was to ensure that observed differences among character states were not dependent on the topology/branch lengths selected as bounded subtrees. Supporting Information 1 provides more details of these analyses.

State-dependent analyses were performed on the Hydra supercomputer provided by the facilities of the Laboratories of Analytical Biology (LAB) of the National Museum of Natural History, Smithsonian Institution. Appendices S1 and S2 are available at https://github.com/isabelsanmartin/Trait-dependent-analyses-Meloinae.

3 | RESULTS

3.1 | Sequencing, assembly and mitogenome organization

A total of 14 complete and seven partial mitogenomes of 21 species of Meloidae were newly generated in our study. The number of reads, mean coverage, length of each mitogenome, base composition and primers are provided in Table S1, Table S3 and Appendix S1. Genome organization was shared across all complete sequenced mitogenomes, and followed the molecular organization described by Du et al. (2016, 2017). The circular genome, represented in Figure S7, encoded for 13 protein-coding genes, 2 rRNAs and 22 tRNA genes, and also contained a putative control region. Major strand encodes genes: *cox1*, *cox2*, *cox3*, *cytB*, *ATP6*, *ATP8*, *NAD2*, *NAD3*, and *NAD6*; as well as the following tRNAs: *L2*, *K*, *D*, *G*, *A*, *R*, *N*, *S1*, *E*, *T*, *S2*, *I*, *M* and *W*. Minor strand encodes genes: *NAD1*, *NAD4*, *NAD4L*, *NAD1* and ribosomal *16S* and *12S*; as well as the following tRNAs: *F*, *H*, *P*, *L1*, *V*, *Q*, *C* and *Y*.

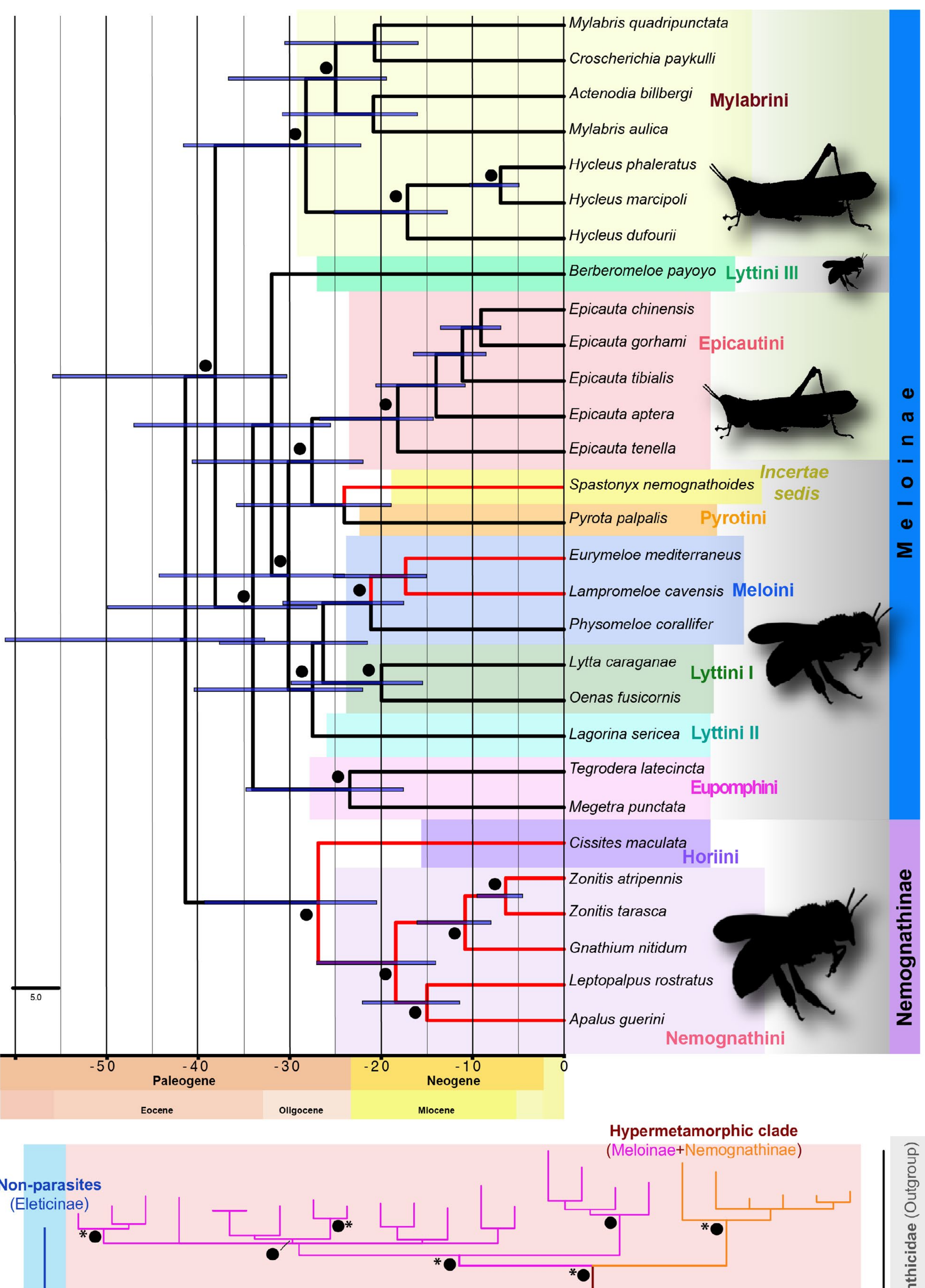
3.2 | Phylogenetic inference

Tree topologies obtained from the analyses of the mitogenome data set using different coding strategies and inference frameworks, were largely congruent, especially for the backbone basal nodes (Figure 4, Figures S1, S8-S10). The RAXML and the MrBayes phylogenies based on the NT- and AA+rNT matrices, supported subfamilies Nemognathinae and Meloinae as monophyletic sister groups, with strong clade support (PP = 1/ bootstrap support (BS) = 100; Figures S8–S9). Within Nemognathinae, the genus *Cissites*, representing the tribe Horiini, was placed as sister to all included representatives of Nemognathini, again with strong support (PP = 1/BS = 100). The first splitting event within Meloinae separates the tribe Mylabrini, recovered as monophyletic (1/100), as sister to a clade that includes all remaining taxa (1/89). Within this larger clade, relationships among tribes and genera were not fully resolved (0.6/28, Figure 4, Figures S8-S9). The tribes Eupomphini and Epicautini were also recovered as monophyletic with maximum clade support (1/100). Phylogenetic relationships with other families of Tenebrionoidea were also congruent across trees, showing Anthicidae as sister to Meloidae (here represented by the hypermetamorphic clade), with Mordellidae as their sister-group (Figures S8-S9). The PhyloBayes analysis with the CAT-GTR model did not converge (results not shown); the simpler CAT-Poisson model recovered a topology that was largely congruent with the RAXML and MrBayes trees, but with lower resolution for intertribal relationships (PP < 0.5, Figure S10). Bayes Factor comparisons of the unconstrained and constrained analyses rejected the hypothesis of Epicautini and Mylabrini as sister tribes (H_0) in favor of the alternative hypothesis (H_1, the two tribes do not form a monophyletic group), with very strong support (Kass & Raftery, 1995): 2lnBF = 2*((−93393.74)-(−93407.19)) = 26.9.

The tree topology derived from the MT+NC data set (Figure 4-inset, Figure S11) was congruent with the trees obtained from the mitogenomic analyses for the backbone basal nodes (Figure 4, Figures S8–S10). There was also consistency in major relationships between the MT-NC and the nuclear-only phylogeny (Figure 4-inset). They both recovered the monophyly of Meloidae with strong clade support (posterior probability (PP) = 1. Within Meloidae, subfamilies Meloinae and Nemognathinae conform a monophyletic group that excludes the representative sample of the nonparasitic Eleticinae (PP = 1). Monophyly of the tribes Mylabrini, Eupomphini, Epicautini and Pyrotini within Meloinae, and of Nemognathini and Horiini within Nemognathinae, were also recovered in the MC-NT tree with high support (PP = 1). Discordant nodes receiving significant clade support (PP >0.9) were mainly those concerning generic or species-level relationships (Figure 4-inset). In general, the MT+NC and the nuclear-only trees provided lower resolution at those phylogenetic levels than the mitogenomic data set (Figure S11) (Figure 4-inset).

3.3 | Divergence time estimation

Estimates of lineage divergence times (MCC tree in Figure 4 and Figure S12) dated the crown-age of the hypermetamorphic Meloidae (Meloinae + Nemognathinae) in the Middle Eocene (Mean 41.42 Ma; 95% HPD 32.61–61.15 Ma). A Late Eocene origin was estimated for the MRCA of the subfamily Meloinae (Mean 38.15 Ma; 95% HPD 30.31–55.96 Ma), whereas the MRCA of Nemognathinae was inferred as Early Oligocene (Mean 28.88 Ma; 95% HPD 20.50–39.60 Ma). Mylabrini also originated in the Early Oligocene according



Mylabris quadripunctata
Croscherichia paykulli
Actenodia billbergi
Mylabris aulica
Hycleus phaleratus
Hycleus marcipoli
Hycleus dufourii
Mylabrini
Berberomeloe payoyo
Lyttini III
Epicauta chinensis
Epicauta gorhami
Epicautini
Epicauta tibialis
Epicauta aptera
Epicauta tenella
Spastonyx nemognathoides
Incertae sedis
Pyrota palpalis
Pyrotini
Eurymeloe mediterraneus
Lampromeloe cavensis
Meloini
Physomeloe corallifer
Lytta caraganae
Lyttini I
Oenas fusicornis
Lagorina sericea
Lyttini II
Tegrodera latecincta
Eupomphini
Megetra punctata
Cissites maculata
Horiini
Zonitis atripennis
Zonitis tarasca
Gnathium nitidum
Leptopalpus rostratus
Apalus guerini
Nemognathini
Meloinae
Nemognathinae
5.0
-50
-40
-30
-20
-10
0
Paleogene
Neogene
Eocene
Oligocene
Miocene
Hypermetamorphic clade
(Meloinae+Nemognathinae)
Non-parasites
(Eleticinae)
Anthicidae (Outgroup)
8.0

FIGURE 4 Mitogenomic phylogeny of hypermetamorphic Meloidae. Chronogram with estimated divergence times between lineages obtained in BEAST using relaxed molecular clocks and the mitogenomic data set. The translated amino acid sequences for the coding genes +the non-coding ribosomal genes matrix (AA+rNT-matrix) was used for this analysis. The figure shows the maximum clade credibility tree with mean ages and 95% high-posterior density credibility intervals, represented by the purple horizontal bars; gray circles indicate nodes receiving clade support with a posterior probability value (PP)>0.95 in the MrBayes analysis and a bootstrap (BS) value above 70 in the maximum likelihood RAXML analysis (see text). Left colour shades represent different tribes; right colour shades represent the characteristic host of each tribe next to its clade; red colour branches represent phoretic lineages. The phylogeny shown at the bottom was generated in MrBayes with the concatenated mitochondrial-nuclear matrix (16S + wingless + 18S) to explore the monophyly of the hypermetamorphic clade (Meloidae + Nemognathinae); the black dots represent well-supported clades (PP > 90) by the combined mitochondrial-nuclear data set; the asterisks represent well-supported clades in the nuclear-only data set

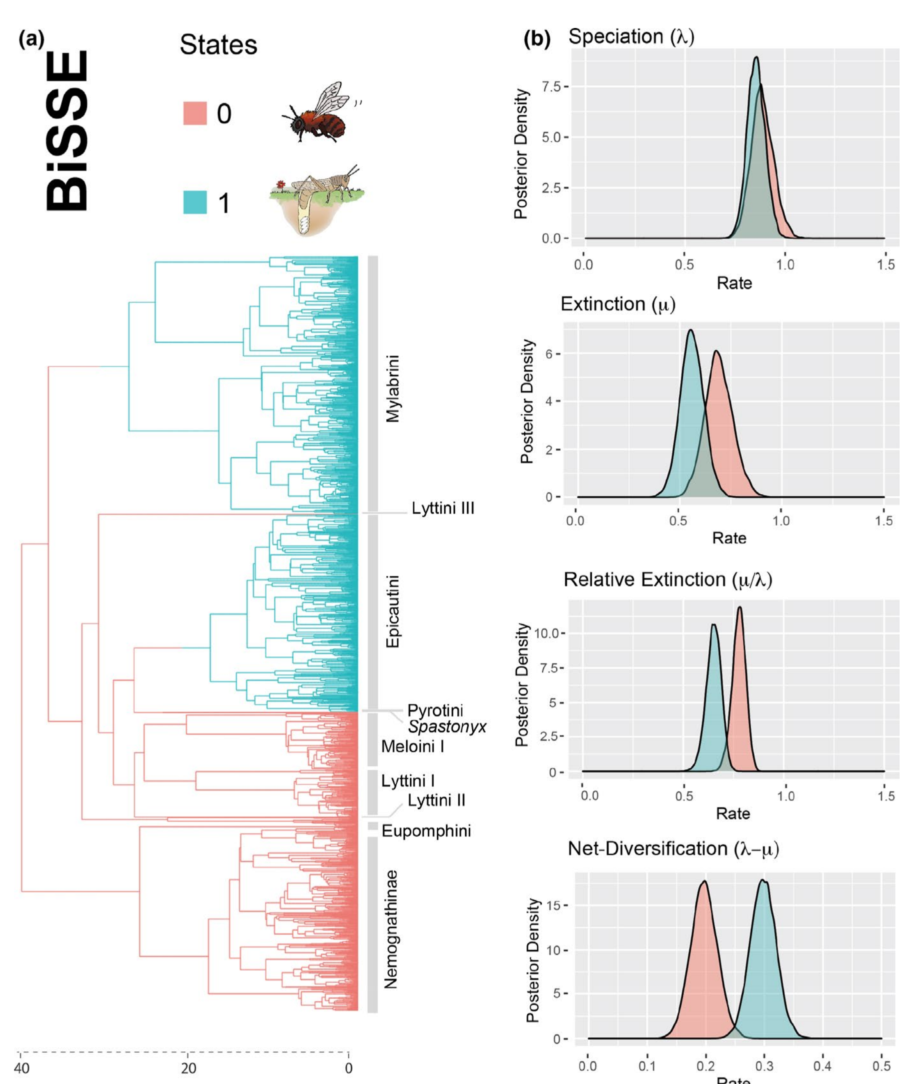


FIGURE 5 Maximum a posteriori reconstruction of host choice evolution in Meloidae and trait-dependent posterior distributions of diversification rates estimated through BiSSE. (a) Host choice evolution simulated under Bayesian stochastic character mapping; divergence times in millions of years are indicated by the axis at the bottom of the tree; branch colours denote different host; transitions between character states are indicated by changes in colour along the branches; note that the state 0 (bee-parasitoid) is reconstructed as the ancestral state of the hypermetamorphic Meloidae and also as the ancestral state of each family. (b) Posterior densities of speciation (λ), extinction (μ), relative extinction (μ/λ) and net-diversification (λ–μ) rates. Colours correspond to the posterior probabilities for a given state; changes in host choice from state 0 to 1 are associated with diversification rate heterogeneity. Bee parasitoids lineages show higher speciation and extinction rates than grasshopper specialists, thus grasshopper specialists' lineages are associated with the higher diversification rates and lower relative extinction rates than bee parasitoids

to our estimates (Mean 28.23 Ma; 95% HPD 22.23–41.61 Ma); the MRCAs of the remaining tribes of Meloinae and Nemognathinae originated in the Early Miocene, with age estimates ranging between 23 and 18 Ma (Figure S12).

3.4 | State dependent diversification

SSE analyses with BiSSE using phoretic behaviour as the focal character, did not show differences in speciation or extinction rates among states (Figure S13). SSE analysis with BiSSE using host type as focal character (Figure 5) supported similar speciation rates but higher extinction rates in bee-parasitoids (state 0) compared to clades feeding on grasshopper eggs (state 1). This resulted in net diversification rates and relative extinction rates that were lower and higher, respectively, in bee-parasitoids compared to grasshopper-parasitoid clades. There was some overlap between the marginal posterior distributions of the extinction rates across states (Figure 5). However, pairwise comparisons of values across the MCMC posterior set (Figure S14) produced a distribution of differences (s0–s1) in which the 95% credibility interval was larger or smaller than zero (i.e. versus overlapping zero), indicating significant differences in extinction

rates between the bee- and grasshopper specialists (Figure S14B). This difference was not significant for the speciation rates (Figure S14A), however, suggesting that differences in net diversification rates between the two states (Figure 5b) are caused by heterogeneity in extinction rates. The transition rate from bee-parasitoidism towards grasshopper specialization (0 to 1) was higher than in the other direction (1 to 0), with marginal statistical significance (Figure S14). Marginal character reconstructions of ancestral states in the MAP tree (Figure S15A) showed that the ancestral condition for the MRCA of Nemognathinae and Meloinae was a bee-parasitoid. Stochastic character mapping showed two independent events of host-jump from bees to grasshoppers, along the branches subtending the MRCAs of tribes Mylabrini and Epicautini (Figure 5); posterior probabilities were high for these transition events (Figure S15B).

The HiSSE model, allowing for the existence of correlated hidden traits (Figure 6) supported a similar pattern: the net diversification rate and the rate of relative extinction were lower and higher, respectively, for bee-parasitoids than for grasshopper specialists, though this was caused by differences in the rates of speciation. Differences in the rates of diversification between the observed states 0 and 1 were maintained within each hidden state (i.e., between 0A and 1A and between 0B and 1B, Figure 6). However, for some parameters there was overlap between the marginal posterior distributions of opposite joint observed*hidden states, for example between 1A and 0B, indicating a strong effect of the hidden trait (Figure 6). Moreover, reconstruction of ancestral states and transition events in the MAP tree (Figure 6, Figures S16A, Figures S16B) suggested phoresy as the "hidden trait" coevolving with host specificity: for example, lineages that behave as phoretic parasitoids of bees were reconstructed as 0B (e.g., Nemognathinae, *Meloe*), while nonphoretic parasitoids of bees were inferred as 0A (e.g., Pyrotini, Eupomphini). The state of the most recent common ancestor (MRCA) of subfamilies Nemognathinae and Meloinae was reconstructed as 0A, as well as the MRCA of each subfamily (Figure 6). Intriguingly, the branch leading to genus *Cissites*, a phoretic parasitoid on bees, as well as the ancestor of tribe Nemognathini, were reconstructed as 0A (Figure 6); however, marginal posterior probabilities were lower on these long branches, indicating uncertainty in the stochastic character mapping (Figure S16B).

The MuSSE analysis, with phoresy and host specificity modeled together (Figure 7), found significant differences in extinction rates between bee- and grasshopper-parasitoids, but only for the nonphoretic bee lineages. Thus, nonphoretic bee-parasitoids (state 1) were associated with significantly higher extinction rates than phoretic bee-paraitoids (state 0) and grasshopper specialists (state 2), while speciation rates were similar among the three states (Figure 7). This resulted in significantly higher relative extinction rates and lower diversification rates for clades with state 1 compared to those associated with states 0 or 2. In contrast, there were no significant differences (i.e., overlapping marginal distributions for all parameters) between phoretic bee-parasitoids and grasshopper specialists (Figure 7). Similarly, pairwise comparisons of transition rates among the three focal states, phoretic (0) and nonphoretic (1) bee-parasitoidism, and grasshopper specialization (2), showed no significant differences. Reconstruction of ancestral states (Figure S17) and stochastic character mapping (Figure 7) supported nonphoretic bee-parasitoidism as the ancestral state for the MRCA of Nemognathinae and Meloinae. Transitions from nonphoretic bee-parasitoids towards phoretic bee-parasitois or grasshopper-host specialization, took place in the hypermetamorphic Meloidae in at least five independent events: the MRCAs of subfamily Nemognathinae and tribes Meloini I, Meloini II, Mylabrini, and Epicautini. A sixth event is observed in *Cissites*, which starts as an ancestrally nonphoretic bee parasitoid but shifts to phoretic bee-parasitoid at circa 11 Ma along the branch (Figure 7); marginal support for this shift was low (Figure S17). Introducing hidden traits in this model (MuHiSSE) did not change these results, with hidden state B showing larger differences among the focal states than hidden state A (Figure S18). Our MuSSE inferences were also robust to the choice of subtrees for the empirical backbone-simulated phylogeny: pairwise comparisons across the 100 empirical-simulated phylogenies showed a distribution of values with a mean centred on 0 (i.e., no significant differences, Figure 8).

All SSE analyses reached convergence, with trace plots showing adequate mixing and ESS values larger than 200 for all parameters (for many, >1000) except for MuHiSSE. Therefore, this analysis was excluded from model comparison with Bayes Factors. The marginal likelihood for each model is shown in Table 2. The best model was HiSSE (ps = −16904.29), followed by MuSSE (ps = −16925.32) and BiSSE (ps = −16950.15); CID2 showed the least support from the data (ps = −16956.41).

4 | DISCUSSION

4.1 | Life strategy evolution in Blister Beetles

Our nuclear-mitochondrial phylogeny (Figure 4, Figure S11) concurs with previous studies in placing the free-living, nonparasitoid subfamily Eleticinae as the sister-group of the parasitoid Meloidae, that is, the clade formed by the reciprocally monophyletic subfamilies Nemognathinae and Meloinae (Bologna et al., 2008; Bologna & Pinto, 2001). Our SSE results (Figure 5) also supports previous hypotheses that considered bee-parasitoidism as the primitive life strategy of the hypermetamorphic clade of Meloidae (Bologna et al., 2008; Bologna & Pinto, 2001). Molecular dating places the origin of this strategy, the crown-node of Meloinae and Nemognathinae, in the Middle Eocene (Figure 4; Figure S12). Bee-parasitoidism was retained by all but two of the hypermetamorphic Meloidae clades, the nonsister tribes Mylabrini and Epicautini, which independently switched from bees to grasshopper hosts (Figure 5). Stochastic character mapping (Figure 5, Figure S12, Figure S14-S15) indicate that the first host jumping event occurred in the Early Miocene along the stem-branch of Mylabrini, while the second host jump, along the stem-branch of Epicautini, took place 10 million years later, during the Mid-Miocene. Our mean age estimates for the hypermetamorphic

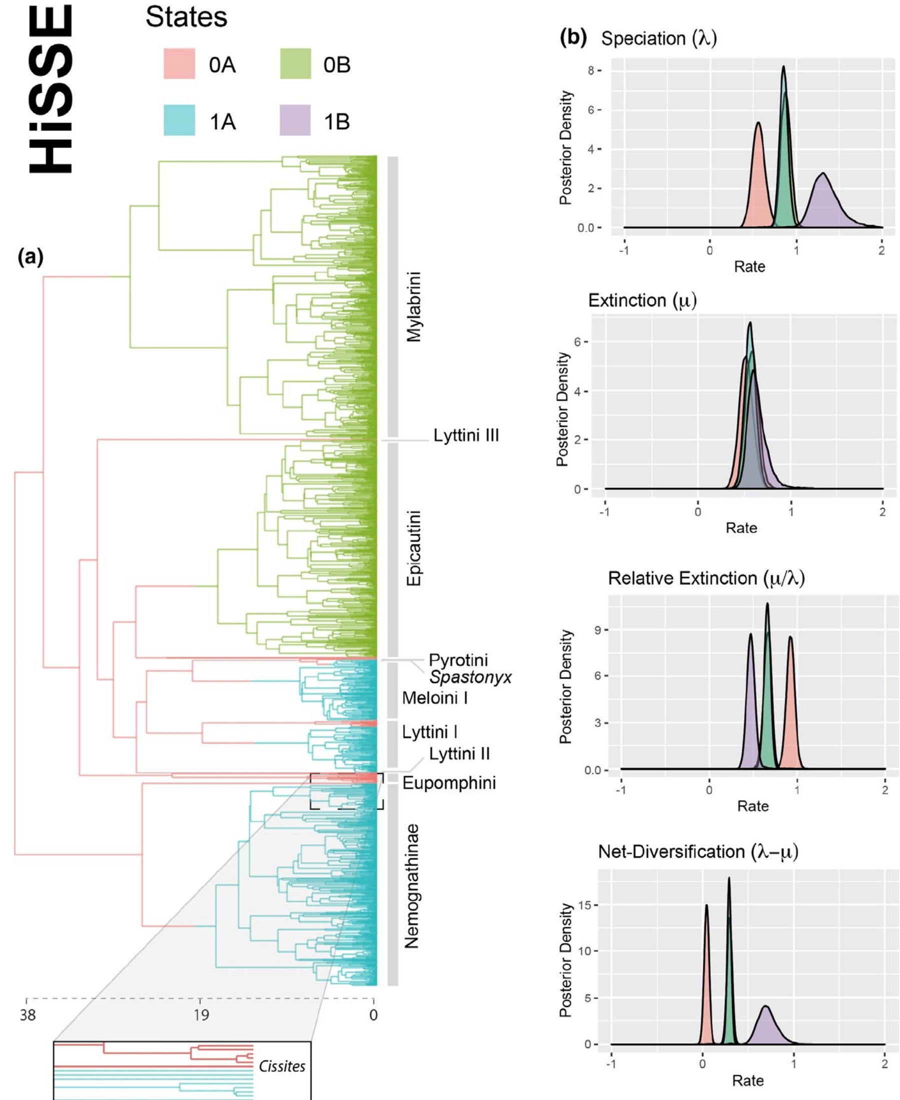


FIGURE 6 Maximum a posteriori reconstruction of host choice evolution in Meloidae and trait-dependent posterior distributions of diversification rates estimated through HiSSE. (a) Host choice evolution simulated under Bayesian stochastic character mapping; divergence times in millions of years are indicated by the axis at the bottom of the tree; branch colors denote the four different states, being 0 and 1 the observed states (bee parasitoid and grasshopper specialists, respectively) and a and b the hidden states; transitions between character states are indicated by changes in colour along the branches; note that lineages reconstructed as 0B coincide in most cases with the phoretic lineages, such as Nemognathinae and Meloini II, while nonphoretic parasitoids of bees, were reconstructed as 0A such as Lyttini II and Pyrotini; inset panel is signaling that the phoretic genus *Cissites* was reconstructed as 0A while the rest of the subfamily Nemognathinae was reconstructed as 0B. (b) Posterior densities of speciation (λ), extinction (μ), relative extinction (μ/λ) and net-diversification (λ–μ) rates. Colours correspond to the posterior probabilities for a given state; note that the posterior densities of speciation, relative extinction and diversification rates are in partial agreement with BiSSE results; however, the overlapping of the marginal posterior distributions for states 1A and 0B indicates that the background rate changes are unassociated with the trait in question: host-type

clade Nemognathinae-Meloinae (41.4 Ma) are older and younger, respectively, than those estimated for the crown-age of the family Meloidae by McKenna et al. (2015): 37 Ma, and Toussaint et al. (2017): 65 Ma (McKenna et al., 2019 only estimated the age of stem Meloidae). Because none of those studies included Nemognathinae, our estimates are not comparable to theirs (we are estimating a less inclusive clade, one node down). However, even if the crown age of the parasitoid clade of Meloidae turns out to be older or younger than our estimates, we do not think that this invalidates our SSE results, as these analyses consider relative differences in clade age. Moreover, our simulation approach did contemplate the large uncertainty in mean age estimates for the basal nodes. For example, the 95% HPD for the crown-age of the hypermetamorphic meloids estimated here (61.2–32.4 Ma) partly overlaps with the Toussaint et al. (2017) credibility interval for the crown-age of Meloidae (one node up. 83.6–44.5 Ma), and fully overlaps with the confidence interval of McKenna et al. (2015) for the same node (56–20.4 Ma), indicating high uncertainty in the molecular clock.

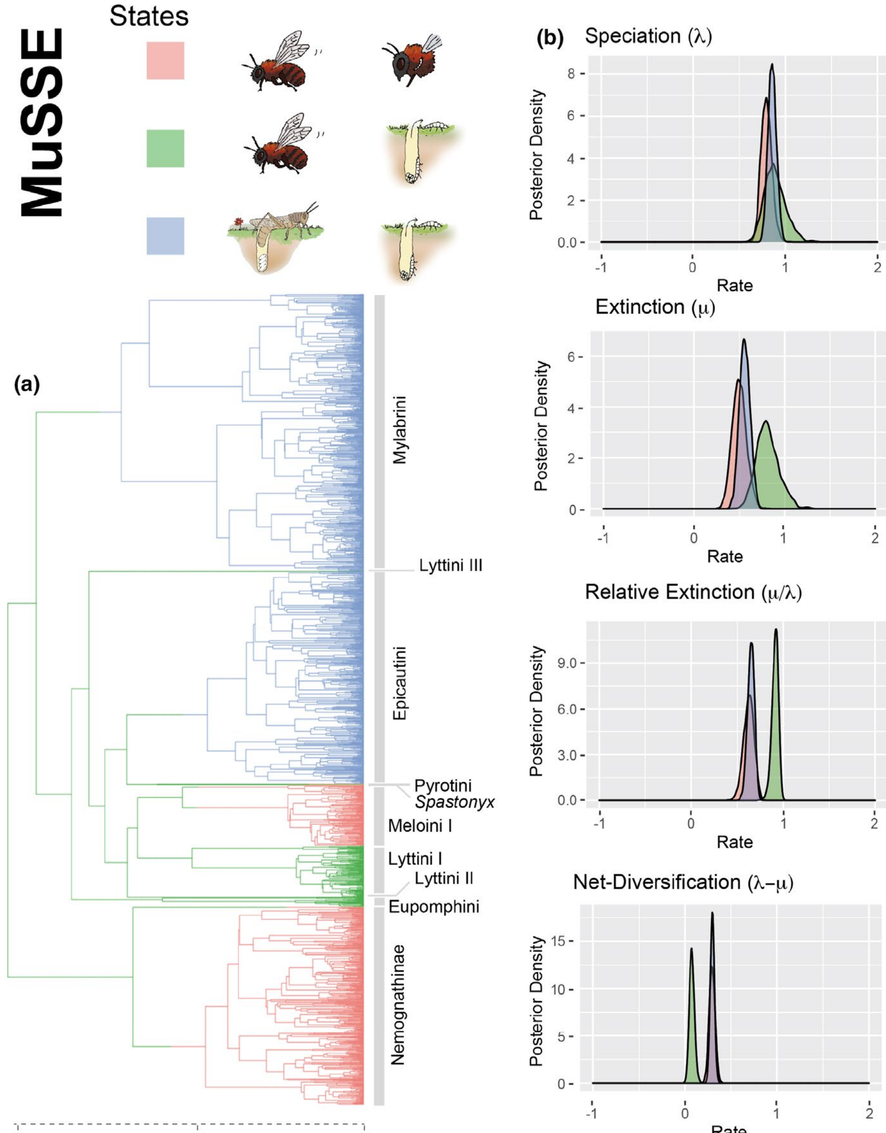


FIGURE 7 Maximum a posteriori reconstruction of host choice evolution in Meloidae and trait-dependent posterior distributions of diversification rates estimated through MuSSE. (a) Life strategy evolution simulated under Bayesian stochastic character mapping; divergence times in millions of years are indicated by the axis at the bottom of the tree; branch colors denote different life strategies; transitions between character states are indicated by changes in colour along the branches; note that a nonphoretic parasitoids of bee nest is reconstructed as the ancestral state of the hypermetamorphic Meloidae and also as the ancestral state of each family. (b) Posterior densities of speciation (λ), extinction (μ), relative extinction (μ/λ) and net-diversification (λ−μ) rates. Colours correspond to the posterior probabilities for a given state; changes in life strategy are associated with diversification rate heterogeneity. The ancestral non-phoretic parasitoids of bees' nests showed the lowest diversification rates and the highest relative extinction rates, while there was no significant difference neither in diversification rates nor relative extinction rates between phoretic parasitoids of bee nest and parasitoids of grasshoppers

Phylogenetic evidence for the homoplasy of the host jump to grasshopper eggs is congruent with previous phylogenies based on morphology or molecular evidence (Bologna et al., 2008; Bologna & Pinto, 2001). Although there is ample evidence that host changes are relatively frequent in nature (Forbes et al., 2017; Giraud et al., 2010; Johnson et al., 2011; Sorenson et al., 2003; Wolfe et al., 2007; Zhang et al., 2020), "dramatic" host-jumps, i.e. jumps to a new host from a different family, order or phylum, are evolutionarily "rare" compared to host shifts towards species or genera closely related to the original host (Braga et al., 2020; Engelstädter & Fortuna, 2019; Foster, 2019). Establishing a sustainable relationship with a new host species represents an important challenge for parasites, which might require new morphological or physiological adaptations (Engelstädter & Fortuna, 2019), or even genetic modifications (Tanaka et al., 2019). In other words, host-switch success will depend on the phylogenetic distance between the original and new hosts (Braga et al., 2020; Engelstädter & Fortuna, 2019; Foster, 2019).

Host change in Mylabrini and Epicautini represents an example of a "dramatic" host-jump. Insect orders Hymenoptera and Orthoptera are phylogenetically distant in Hexapoda, with their MRCAs separated by more than 350 Ma (Misof et al., 2014; Song et al., 2015). Additionally, grasshoppers and bees exhibit very different development strategies, and their eggs can differ markedly in chemical composition and eggshell structure (Hilker & Meiners, 2008); for instance, some species of acridids exhibit a complex chorion (the thickest part of the eggshell) layer (Viscuso et al., 1990). One explanation for the success of a host jump from Hymenoptera to Orthoptera is ecological opportunity. Grasshoppers in the family Acrididae are a dominant component of biodiversity in grassland ecosystems (Badenhausser et al., 2009; Baldi & Kisbenedek, 1997), where species of Mylabrini and Epicautini are also abundant (Bologna & Pinto, 2002; Pinto, 1991; Pinto & Bologna, 1999). Given that the Orthopteran hosts and parasitoid meloids share the same biome, and that food resources, acridid egg-pods, are abundant in grasslands, one could envisage continuous attempts by parasitoids to shift to the new host until a successful jump was achieved, not once, but twice independently over the evolutionary history of Meloinae. Another key aspect in the success of a host jump from

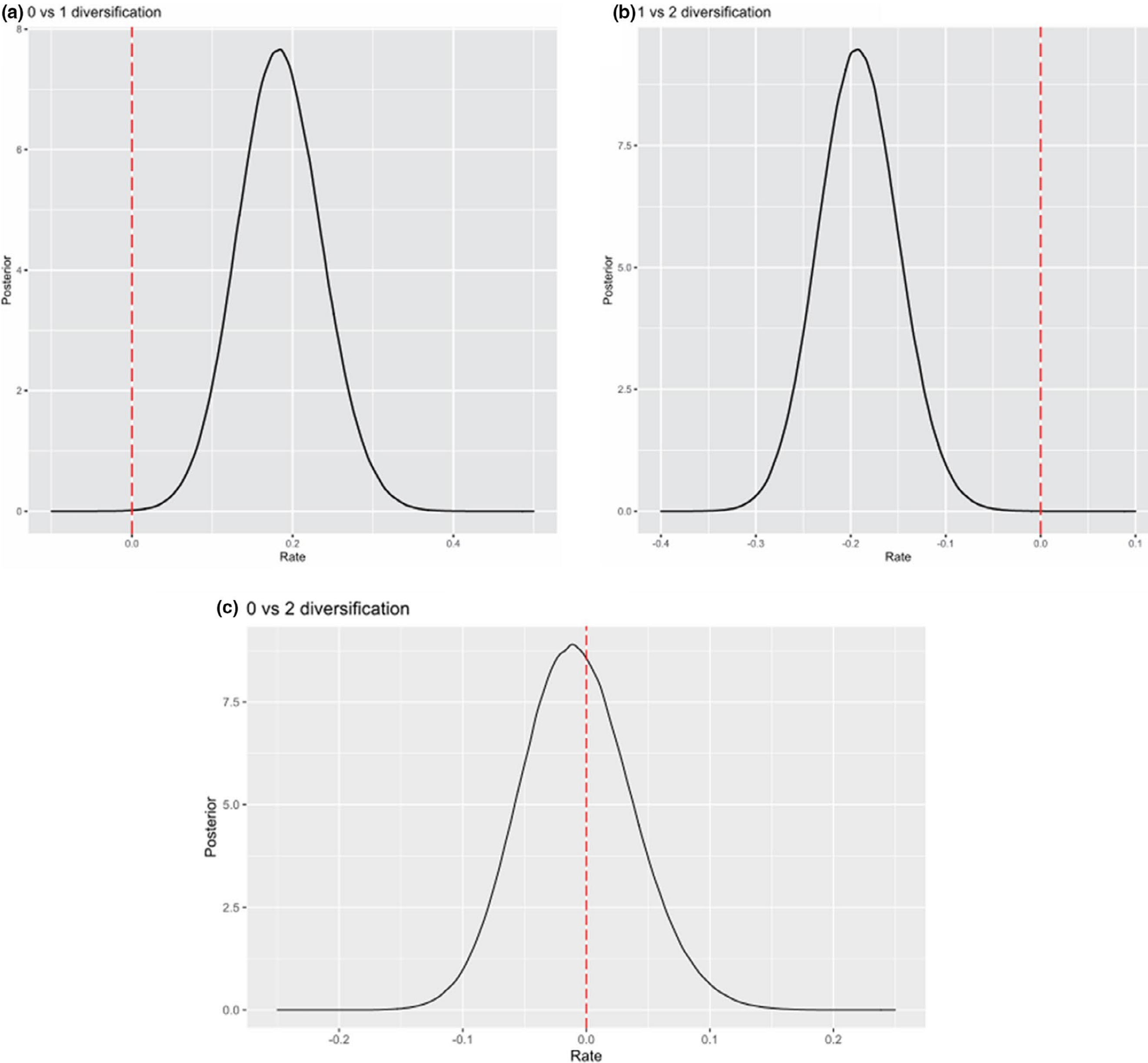


FIGURE 8 Phylogenetic uncertainty analysis. Plot of pairwise differences of diversification rates among states (0, 1, 2) estimated by the MuSSE model. Histograms represent the distribution of these values across the 100 backbone+simulated subtrees phylogenies. The distribution is centred in 0 (represented by the red line) for comparison between states 0 and 2 (c) (i.e., there are no differences in diversification rates across the simulated phylogenies). However, there are significant differences for the other two pairwise comparisons (0 vs. 1 and 1 vs. 2) (a, b), confirming the results reported in Figure 5

bees to grasshoppers is probably the hypermetamorphic life cycle of the parasitoid Meloidae, which imposes strict physiological demands and limits the choice of potential hosts. A regular life cycle of a species in Nemognathinae or Meloinae involves multiple larval stages, and spans a minimum length of one year (Figures 1, 2) (Horsfall, 1941; MacSwain, 1956; Selander & Mathieu, 1964; Selander & Weddle, 1969). Stable temperature and soil moisture are probably the main factors governing larval survival (Erickson & Werner, 1974; Zhu et al., 2006). As in the case of the bee nests, acridid egg-pods (i.e., a chamber in the ground) meet the requirements of a stable environment with constant temperature and humidity, in which the successive larval stages can complete their development and emerge as adults after one or several years. This "ecological match" between the old and new hosts (bees and grasshoppers) can explain why meloids have not parasitized other insects equally abundant in the grassland biome, such as beetle species from the families Tenebrionidae and Chrysomelidae. Di Giulio et al. (2003) mentioned larvae of *Cyaneolytta* (Meloinae) as being phoretic on Carabidae, but the feeding habits of this genus are unknown. Ecological "fitting" between hosts and parasites as a factor for host jump success has been described in other parasitoid insects; for example, velvet ants (Hymenoptera: Mutillidae) have been able to shift hosts from Hymenoptera to ant nest-dwelling Coleoptera with enclosed larvae (Chrysomelidae: Clythrini; Brothers et al., 2000).

TABLE 2 Log-marginal likelihood values estimated with the path sampling (PS) and stepping-stone (SS) methods for each trait-dependent diversification model applied in this study: BiSSE (Figure 5), HiSSE (Figure 6), MuSSE (Figure 7), CID2 (Figure S6)

SSE MODEL	Marginal likelihood SS	Marginal likelihood PS
BiSSE	−16950	−16950.15
HiSSE	−16904.28	−16904.29
MuSSE	−16925.53	−16925.32
CID2	−16956.29	−16956.41

Host-jump was not the only change in the evolution of the hypermetamorphic Meloidae. A change in the locomotion mode used by the first instar larvae to reach the bee-nest, from active crawling over ground to latching to a passing bee, occurred more than once along the evolution of Meloinae, and, according to the MuSSE results, also within the sister-tribe Nemognathinae (Figure 7, Figure S16). This inference agrees with previous studies, who pointed out that bee-parasitoidism and nonphoresy were the ancestral states of the hypermetamorphic meloids, and that phoresy evolved at least twice within subfamily Meloinae (Bologna et al., 2008; Bologna & Pinto, 2001). The homoplastic nature of phoresy is evidenced in the current taxonomy: our results demonstrate that traditional tribes defined as phoretic, such as Meloini, or nonphoretic such as Lyttini, are nonmonophyletic (Figure 4).

Host-phoretic "specialization", in which the parasite modifies certain traits to ensure successful latching to a specific host, has been described in mites (Athias-Binche et al., 1993; Brown & Wilson, 1992). In Meloinae, some lineages such as *Meloe* exhibit large morphological diversity in larval traits related to phoresy, including variable development of a pygopod to crawl on vertical surfaces, or a variable degree of abdominal sclerotization (Bologna, 1983, 1991; Bologna et al., 2008; Bologna & Pinto, 2001; Cros, 1940; Kaszab, 1969; MacSwain, 1956; Pinto & Selander, 1970; Sánchez-Vialas et al., 2021; Selander, 1964). Conversely, a shift towards an Orthopteran host does not seem to have involved any major change in morphology or physiology (i.e., no set of unique morphological traits are shared by the larvae of Mylabrini and Epicautini, Bologna & Pinto, 2001), unlike in examples of host jumping in other insects (Turrisi & Vilhelmsen, 2010). This agrees well with the concept of "ecological fitting" (Janzen, 1985), in which the formation of a new interaction does not require the evolution of novel traits; instead, traits developed for previous host-parasite interactions can be "co-opted" for a new interaction given the right conditions (Agosta, 2006).

4.2 | Host-jump and phoresy: Historical contingency and evolutionary dead-ends in Meloidae

Our BiSSE analysis shows that the grasshopper specialist tribes Mylabrini and Epicautini, which are by far the most speciose, exhibit higher speciation rates than other bee-parasitoid lineages within Meloinae (Figure 5, Figure S17). Evidence that host specialization can be a powerful driver of diversification comes from many different organisms (Ehrlich & Raven, 1964; Forbes et al., 2017; Giraud et al., 2010; Johnson et al., 2011; Sorenson et al., 2003; Zietara & Lumme, 2002). Adaptation to a new habitat (the host) can induce reproductive barriers in a relatively small number of generations (Hendry et al., 2007), and thus accelerate the rate of speciation events, sometimes leading to patterns consistent with adaptive radiations (Bush et al., 2019; Farrell & Sequeira, 2004; Forbes et al., 2017; Fordyce, 2010; Karvonen & Seehausen, 2012; Zietara & Lumme, 2002).

However, host jumping alone is not responsible for the striking differences in species richness between subfamilies, tribes and genera of parasitoid Meloidae. Although BiSSE detected a signal of trait-diversification dependency with host-type (Figure 5), HiSSE and MuSSE (Figures 6, 7) suggests that this trait alone cannot explain the observed rate heterogeneity; these results were robust against phylogenetic uncertainty (Figure 8). Our SSE analyses indicate that phoretic bee-parasitoid lineages exhibit higher net diversification rates and lower relative extinction rates than nonphoretic bee-parasitoid lineages, with the latter being the ancestral condition (Figures 6–7; Figure S16). Bologna et al. (2008) suggested that phoresy was evolutionarily advantageous in Meloidae because it enhances the ability of the first instar larvae to reach the host, thus ensuring food resource availability. Actively latching to the host, rather than wandering around to locate the nest, can also be seen as a strategy to save energy (Baumann et al., 2018). Phoresy may also drive speciation through host-phoretic specialization or as a strategy to ensure panmixia (Opatova & Šťáhlavský, 2018). In sum, our SSE analyses demonstrate that the evolution of phoresy contributed to accelerated diversification rates within the hypermetamorphic Meloidae, and that the two traits, host type and phoretic behaviour, are needed to explain the current pattern of species richness (Figures 6, 7, Figures S17-S17): no significant differences in net diversification or relative extinction rates were found between phoretic bee-parasitoids and grasshopper parasitoids (Figure 7, Figure S17).

While trying to understand why certain groups diversify more than others, the idea of a single key factor promoting elevated diversification rates, for example, the evolution of a morphological innovation, the invasion of a new isolated environment, or the effect of a mass extinction event depleting extant diversity, has been a dominant one in the literature (De Queiroz, 2002; Donoghue, 2005; Hodges & Arnold, 1995; Hunter & Jernvall, 1995). Yet, despite numerous recent studies testing for an association between trait evolution and diversification, few of them have found evidence of a single trait driving a shift in diversification rates (Condamine et al., 2018; Lagomarsino et al., 2017; Moharrek et al., 2019). Instead, the dominant pattern is one in which bursts of diversification are explained by the confluence of multiple factors, sometimes acting at unison (Donoghue & Sanderson, 2015), sometimes in a sequence (Donoghue, 2005), or contingent upon one another (Givnish et al., 2014).

Our study supports the idea that the parallel innovations brought about by phoresy and host-jump acted as diversification triggers in

the parasitoid Nemognathinae and Meloinae. However, these two traits did not act simultaneously or synergistically, as in the concept of "synnovations" (Donoghue, 2005; Donoghue & Sanderson, 2015). Instead, phoresy acted as a diversification driver through host-specificity. BiSSE showed that the evolution of phoresy alone did not result in events of accelerated speciation (Figure S13); it was only when analysed together with host-jump, as part of a multi-state character, that we could detect significant differences in diversification rates among clades (Figure 7). This is because in Meloidae there are only phoretic/nonphoretic bee-parasitoids and nonphoretic grasshopper-parasitoids. Phoretic grasshopper parasitoids are an improbability in nature because adult grasshoppers never return to the egg-pods once the oviposition takes place. In contrast, the social behaviour of bees, characterized by long-term offspring rearing, makes phoresy a highly efficient strategy to ensure food resources for lineages that are bee parasitoids (Danforth, 2007). Therefore, all phoretic lineages in Meloidae are bee-parasitoids. This “contingency” between the onset of phoresy and host jump can be seen in the results of the HiSSE analysis: though the joint observed*hidden state 1B is associated with the highest speciation rates and lowest extinction rates (Figure 6b), it does not appear in any of the marginal reconstructions of ancestral states (Figure 6a) or transition events along branches (Figure S15A). These puzzling results fit the so-called “Bayesian fair-balance paradox” (Autzen, 2018; Bengt, 2018): when two hypotheses are equidistant from the truth, or the set of hypothesis being examined does not include the true one, Bayesian model selection can support one of them with high posterior probability; in fact, repeating the HiSSE analyses multiple times resulted in some reconstructions showing 1B, instead of 1A, as the ancestral state of Mylabrini and Epicautini. In other words, in our analysis setting, states 1A and 1B are equally likely (or unlikely) because there is only one state (1) for grasshopper-parasitoids, whereas bee-parasitoids can be either nonphoretic (0A) or phoretic (0B) lineages; see Supporting Information 1 for a detailed explanation. If this is the case, phoresy in Meloidae can be seen as an example of causal contingency (Beatty, 2006): a change on a trait that is “contingent upon” a previous change on a different trait, which either promotes or restrains the latter change. So far, SSE models have never been used to explore contingency in host specialization. Our HiSSE and MuSSE analyses indicate that phoresy was contingent to host specificity in Meloinae: once a lineage jumped towards a Orthopteran host, phoretic behaviour was not a possible outcome.

From the above, it can be deduced that the two derived strategies in Meloinae, phoresy and grasshopper-specialization, acted as drivers of rapid speciation or adaptive radiation (Erwin, 1992; Gavrilets & Losos, 2009; Givnish, 2015; Simões et al., 2016), either as an ecological opportunity (i.e., adaptation to a new environment such as the grasshopper egg-pods in Mylabrini and Epicautini), or as a key innovation (i.e., the evolution of the morphological suite necessary for phoresy). In other words, phoresy and host jumps could act as pathways by which Meloidae were allowed to explore new opportunities. This capacity of blister beetles to explore novel evolutionary pathways was also described by López-Estrada et al. (2019) in connection with the rampant morphological disparity exhibited by the species-poor Eupomphini.

Another interesting consequence of causal-dependence in the context of host- parasite associations is the possibility of becoming an "evolutionary dead-end" (Vamosi et al., 2003). This process is generally associated to the acquisition of a character state with high extinction or low speciation rates, or with irreversibility in transition rates (Goldberg & Igic, 2008; Goldberg et al., 2017). Our MuSSE analysis suggest that the ancestral nonphoretic bee parasitoid strategy, characterized by significantly higher extinction rates, behaves as an evolutionary "depauperon", that is, deemed to slowly disappear via extinction or transitions to other strategies (Donoghue & Sanderson, 2015). We found no significant differences in transition rates among the three strategies, but MuSSE (Figure 7) recovered multiple events of transition from nonphoretic bee-parasitoidism towards phoretic bee or grasshopper-parasitoidism, with no reversals, along the approximately 40 million year-evolution of the hypermetamorphic Meloidae. These events were followed by increases in diversification rates fueled by extinction. All Nemognathinae species are phoretic bee parasitoids. However, MuSSE inferred that the ancestor of *Cissites*, a species-poor genus belonging to tribe Horiini, and the ancestor of the sister-tribe Nemognathini, with many more species (Table 1), were nonphoretic bee parasitoids, and that they independently transitioned to phoresy along the long branch leading to each tribe (Figure 7b). It is possible that the high extinction rates associated with nonphoretic bee parasitoidism (Figure 7a), explain why no extant lineage of Nemognathinae exhibits today this life strategy.

In conclusion, we show that nonphoretic bee-parasitoidism was the ancestral life strategy of the hypermetamorphic Meloidae, and that transitions towards phoresy and grasshopper parasitoidism occurred multiple times along the phylogeny, and contributed jointly to the evolutionary success of the parasitoid Meloidae. Our results indicate that these two ecological/key innovations contributed to accelerated diversification rates through a decrease in the extinction rate relative to the nonphoretic bee strategy. Hidden-state SSE models have been used to discard "false positives" when examining a causal association between the evolution of the focal trait and heterogeneity in diversification rates (Condamine et al., 2018; Fernandez et al., 2018; Freyman & Höhna, 2019; Gajdzik et al., 2019; Nakov et al., 2019). We here demonstrate, that these models can also be used to identify an unknown causal force or “hidden” trait (phoresy), whose interaction with the focal trait (host specificity) is driving diversification dynamics within the hypermetamorphic clade of Meloidae; to our knowledge, this other use of HiSSE has never been explored. We also introduce a methodological pipeline to account for low taxon sampling in SSE models, which might be used in higher-level phylogenomic studies.

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AUTHOR CONTRIBUTIONS

Estefany Karen López-Estrada conceived the original idea. Estefany Karen López-Estrada and MGP carried out the fieldwork. JEU, SA, YRJ generated the molecular data. Estefany Karen López-Estrada, JEU and SA assembled and annotated the mitogenomes, and performed phylogenetic inference with help from IS. Estefany Karen López-Estrada and IS designed and conducted the diversification analyses. Estefany Karen López-Estrada wrote the first draft. Estefany Karen López-Estrada and IS wrote the manuscript together with MGP, with contributions from SA and JEU.

CONFLICT OF INTEREST

The authors declare no conflict of interest in this article.

BENEFIT-SHARING STATEMENT

No benefits to report.

DATA AVAILABILITY STATEMENT

DNA assembled sequences: Uploaded to NCBI (OK360633, OK360642, OK360638, OK360628, OK360631, OK360641, OK360635, OK360629, OK360640, OK360626, OK254885, OK254883, OK360643, OK360636, OK360627, OK360634, OK360637, OK360630, OK254884, OK360639, OK360632, MZ161711, MZ161720, MZ161719, MZ161723, MZ161721, MZ161724, MZ161722, MZ161745, MZ161717, MW157952, MW157967, MZ161712, MZ161713, MW157961, MZ161716, MZ161715, MZ161714, MZ161710, MZ161707, MZ161708, MZ161709, MZ161706, MZ161718, MZ161705, MZ161731, MZ161740, MZ161739, MZ161743, MZ161741, MZ161744, MZ161742, MK024580, MZ161737, MW158107, MW158122, MZ161732, MZ161733, MW158116, MZ161736, MZ161735, MZ161734, MZ161730, MZ161727, MZ161728, MZ161729, MZ161726, MZ161738, MZ161725).

R and RevBayes code: All scripts used in this study can be found in Appendix 1 and Appendix 2, which are available at https://github.com/isabelsanmartin/Trait-dependent-analyses-Meloinae

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