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Host conservatism, host shifts and diversification across three trophic levels in two Neotropical forests

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Abstract

Host–parasite systems have been models for understanding the connection between shifts in resource use and diversification. Despite theoretical expectations, ambiguity remains regarding the frequency and importance of host switches as drivers of speciation in herbivorous insects and their parasitoids. We examine phylogenetic patterns with multiple genetic markers across three trophic levels using a diverse lineage of geometrid moths (*Eois*), specialist braconid parasitoids (*Parapanteles*) and plants in the genus *Piper*. Host–parasite associations are mapped onto phylogenies, and levels of cospeciation are assessed. We find nonrandom patterns of host use within both the moth and wasp phylogenies. The moth–plant associations in particular are characterized by small radiations of moths associated with unique host plants in the same geographic area (i.e. closely related moths using the same host plant species). We suggest a model of diversification that emphasizes an interplay of factors including host shifts, vicariance and adaptation to intraspecific variation within hosts.

Introduction

Interactions between trophic levels play a central role in the evolution of biological diversity (Page, 2003; Singer & Stireman, 2005; Thompson, 2005). In particular, host–parasite relationships have figured prominently in our understanding of diversification by providing a framework for investigating the importance of exploitative adaptations and host defences (e.g. Ehrlich & Raven, 1964; Rundle & Nosil, 2005; Becerra, 2007; Matsubayashi *et al.*, 2010). Within this area of evolutionary ecology, research has advanced along at least two separate fronts, one focusing on patterns at a deep temporal and taxonomic scale, and the other focusing on mechanisms driving recent population and species divergence, often with contemporary taxa at an incipient stage of divergence. At the deeper taxonomic level, the emphasis has been on major hosts shifts, for example, among different families of hosts where lineages of parasites adapt to novel resources that subsequently drive adaptive radiations (Ehrlich & Raven, 1964; Schluter, 2000). Evidence for the importance of major host shifts driving diversification comes from a number of groups, such as the colonization of angiosperms by weevils (McKenna *et al.*, 2009), and shifts to new plant families by butterflies (Fordyce, 2010). Along the other major conceptual front in hypotheses of host–parasite diversification is the emphasis on host-switching at the lowest taxonomic levels (Berlocher & Feder, 2002; Drès

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& Mallet, 2002; Funk *et al.*, 2006), in which reproductive isolation between sister species is associated with host-specific adaptations (e.g. Funk, 1998; Lu & Bernatchez, 1999; Nosil, 2004; Forister, 2005; Stireman *et al.*, 2005).

Considering the phylogenetic evidence for the importance of major host shifts and the importance of divergent, host-associated selection at low taxonomic levels, one might conclude that shifts in diet are the major drivers of diversification in herbivorous insects. This could be true if insect diversification is contemporary with host diversification, or temporally lags behind plant speciation (Percy *et al.*, 2004). Futuyma & Agrawal (2009) have cautioned that this conclusion remains unjustified and that the importance of other facets of the parasitic life style, including more complex community interactions, has been insufficiently examined. Winkler & Mitter (2008) surveyed a large number of published phylogenies for herbivorous insects and similarly concluded that the importance of both major (between families) and minor (between species) host switches has been overestimated. Specifically, fewer than half of 145 sister species pairs from 45 phylogenies included different host species. This suggests that other factors, such as historical vicariance, may also play a role in parasite diversification. In fact, more general mechanisms of speciation, such as divergence in allopatry, might interact with ecological processes in diversification (Nosil *et al.*, 2005). The possibility of an interaction between historical and geographical factors and divergence associated with alternate resource use has often been overlooked in discussions of host–parasite diversification.

Although phylogenetic studies of host–parasite relationships are not rare, few studies have targeted highly diverse parasite lineages, particularly at appropriate spatial and temporal scales in which recent divergence could potentially be linked to macroevolutionary trends. Even fewer studies have investigated evolutionary dynamics in a community context across more than two trophic levels (but see Lopez-Vaamonde *et al.*, 2005; Noda *et al.*, 2007; Silvieus *et al.*, 2008; reviewed by Forister & Feldman, 2011). Tropical communities provide species-rich assemblages in which questions regarding resource use and diversification can be addressed. In this study, we examine patterns of diversification in a species-rich tropical moth genus, *Eois* Hübner (Lepidoptera: Geometridae: Larentiinae), its major host plant genus, *Piper* L. (Piperales: Piperaceae), and a group of *Eois*-attacking parasitoid wasps in the genus *Parapanteles* Ashmead (Hymenoptera: Braconidae: Microgastrinae). *Eois* is comprised of roughly 250 described species (Scoble, 1999; Herbulot, 2000) as well as numerous genetically distinct morphospecies (Strutzenberger *et al.*, 2010). Estimates suggest, however, that Neotropical *Eois* richness may be as high as four times the currently described number (Rodriguez-Castaneda *et al.*, 2010). *Eois* species specialize on *Piper* and have narrow diet breadths, with most species feeding on a single or a few *Piper* species (Dyer & Palmer, 2004; Connahs *et al.*, 2009); though, recent evidence suggests that at least some host shifts away from *Piper* have occurred (Strutzenberger *et al.*, 2010). *Piper* is a species-rich genus composed of predominantly understory shrubs that reach their highest diversity in the Neotropics, where over 1000 species are found (Jaramillo & Manos, 2001; Greig, 2004; Quijano-Abril *et al.*, 2006). Because of the high diversity and abundance of *Piper* in the Neotropics, as well as the variety of ecological interactions and chemical defences present in this genus, it has been considered a model for studies of phytochemistry, ecology and evolution (Dyer & Palmer, 2004). At the third trophic level, *Parapanteles* is a potentially large, but still poorly known genus within the diverse microgastrine braconid wasps, with 16 species described from the Neotropics (Valerio *et al.*, 2009). It is likely that there are many undescribed *Parapanteles* species because the majority of Neotropical microgastrines remain undescribed (Smith *et al.*, 2008a; Whitfield *et al.*, 2009). Like all microgastrines, *Parapanteles* are endoparasitoids of lepidopteran larvae and although host associations are known for only a small proportion of the species, they are expected to be highly host specific because high host specialization has been found in a number of microgastrine genera (Smith *et al.*, 2008a).

To investigate the role resource use plays in diversification, we bring together phylogenetic and ecological data for *Piper*, *Eois* and *Parapanteles*. For each host–parasite relationship – *Eois* feeding on *Piper*, and *Parapanteles* feeding on *Eois* – we ask: what is the phylogenetic distribution of host use? If diversification at one level provides the ecological opportunity for diversification at another, then we expect the phylogenetic histories of ecological associates to covary in predictable ways (Page, 2003; Forister & Feldman, 2011). For free-living (as opposed to symbiotic) parasites, such as insect herbivores and parasitic wasps, we do not expect a history of cospeciation to necessarily be manifest as perfectly congruent phylogenetic histories. Instead, the prediction based on cospeciation is for a level of constrained or conserved cladogenesis, in which closely related parasites tend to attack more closely related hosts (Futuyma & Agrawal, 2009). Alternatively, an absence of association between host and parasite phylogenies would raise the possibility that other mechanisms (i.e. biogeographic factors) rather than host-associated ecological divergence have influenced diversification. We address these issues both with tests designed specifically to detect patterns of shared history in host–parasite phylogenies, and with more general methods for exploring the distribution of characters on phylogenies. The present study was not designed to investigate historical factors (e.g. vicariance) directly; though, we conclude with a discussion of future directions that includes such factors.

Materials and methods

To a large extent, the structure of this study was determined by the availability of *Eois* specimens; these specimens came first, then *Piper* specimens were studied as they were hosts for *Eois* caterpillars, and wasp specimens were reared from *Eois* individuals. Differences in how specimens were processed and data were generated are largely due to the unique properties of these different groups as discussed below.

Taxon sampling

Eois

Larvae were collected and reared from two sites, Yanayacu Biological Station (00°36′ S 77°53′ W) in Ecuador, and La Selva Biological Station (10°26′ N 83°59′ W) in Costa Rica. At both sites, 10-m-diameter plots were randomly placed in the forest understory and the stems and leaves of all *Piper* plants were either searched or harvested for *Eois* larvae. Collecting from plots was supplemented with searches on focal *Piper* hosts along trails and roads at both sites. Larvae from each plant were reared at laboratories near collection localities. Specimens were collected from 51 plots in Ecuador representing an elevational range of 50–3200 m. Specimens were collected from 12 plots in Costa Rica, which were all at low elevations. Larval sampling and rearing protocols followed Gentry & Dyer (2002) for general collecting and Rodriguez-Castaneda *et al.* (2010) for collecting in plots.

The genus *Eois* is in need of taxonomic revision and potentially contains hundreds of undescribed species (Rodriguez-Castaneda *et al.*, 2010; Strutzenberger *et al.*, 2010). Thus, for many of the collected specimens, it was not possible to assign a species name. Because of this, larval specimens were assigned a morphospecies name based on morphology when they were collected and, where possible, were identified to species when they emerged as adults.

We developed a sampling scheme that allowed us to address patterns of genetic diversification at multiple levels. We included sequences from individual moths in phylogenetic analyses if they fit one or more of the following criteria: (i) specimens were genetically distinct (unique by at least one base pair that was reliably scored in forward and reverse sequence reads), (ii) specimens could be identified by a unique larval morphospecies name, or (iii) specimens were not genetically distinct, nor could they be identified as morphospecies, but the larvae were found on a unique host plant. This sampling scheme enabled us to investigate even the most incipient levels of diversification within the genus and is consistent with an approach taken by others that emphasizes a continuum of diversity (Mallet, 2008; Scriber, 2010). All *Eois* adult specimens were assigned ID numbers (Table S1) and deposited in collections: Smithsonian Institution (USNM); Ecuador Museum of Natural Sciences (MECN); University of Nevada Reno (UNR).

Piper

The *Piper* species represented in this study include those that were identified as host plants for *Eois* through direct rearing of caterpillars collected in the field. Additional *Piper* species were included to represent the phylogenetic breadth of the group (Jaramillo & Manos, 2001; Jaramillo *et al.*, 2008) and to provide a phylogenetic context for species associated with *Eois*. The majority of sequence data used here for *Piper* come from Jaramillo *et al.* (2008); see Table S2 for specimen details and information on sequences not taken from Jaramillo *et al.* (2008).

Parapanteles

Although a variety of parasitoids have been reared from *Eois* larvae at our sites, we focused on *Parapanteles* because it is one of the most abundant and diverse parasitoid groups in our samples. Parasitoids were reared following Gentry & Dyer (2002) and were identified to genus and provisional morphospecies using a combination of adult and cocoon morphological characters. All *Parapanteles* specimens were deposited at the University of Illinois at Urbana–Champaign following identification and molecular characterization (Table S3).

Establishing trophic connections between parasitoids and a specific caterpillar species is inherently more difficult than documenting caterpillar–plant associations because the caterpillar host is destroyed when the parasitoid emerges. However, we were able to link parasitoids to *Eois* species because our identifications were based on larval morphology before the caterpillar was killed by the wasp. In a small number of cases, *Eois* hosts were not identified, but we retained the parasitoid in our analyses as being reared from '*Eois* sp.' because these specimens contributed to our characterization of the molecular diversity of *Parapanteles* associated with *Eois*.

Molecular methods

DNA was extracted from preserved specimens using the DNeasy Tissue Kit (Qiagen Inc., Germantown, MD, USA). The age of extracted specimens ranged from 6 months to 6 years, with older specimens yielding more degraded DNA. For *Eois*, two loci were amplified: a portion of the mitochondrial gene coding for cytochrome oxidase subunit I (COI) and the nuclear gene coding for translation elongation factor 1-alpha (Ef1-α). For *Piper*, two loci were studied: the nuclear ITS region (including ITS1, 5.8S and ITS2) and a chloroplast region, the *psbJ-petA* intron (see Jaramillo *et al.*, 2008). For *Parapanteles*, one mitochondrial and two nuclear loci were amplified: COI, *wingless* (*Wgls*) and alpha spectrin (ASpec), respectively. For primer sequences and specific protocols, see Table S4. Following amplification,

sequences were generated via Sanger cycle-sequencing using amplification primers (forward and reverse directions) and visualized on an AB1 377 or 3730xl DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA) or a Li-Cor 4200 LongreadIR (Li-Cor Biosystems, Lincoln, NB, USA). Sequences were assembled in BioEdit (Hall, 1999) or SEQUENCHER version 4.10.1 (Gene Codes Corp., Ann Harbor, MI, USA). All sequences were deposited in GenBank (Tables S1, S2 and S3).

Phylogenetic analyses

Correlating patterns of resource use, cospeciation and diversity in our tri-trophic system requires phylogenetic hypotheses for each of the focal groups. We estimated the evolutionary relationships of moth and wasp lineages using Bayesian inference and used the phylogenies of Jaramillo *et al.* (2008) for *Piper*. We evaluated the fit of various models of molecular evolution to each data set with the Akaike information criterion (AIC) in MrModeltest version 2.3 (Nylander, 2004). We then conducted Bayesian analyses using MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003) under these best-fit models.

For *Eois*, the COI data set was partitioned by codon as follows: first position SYM + Γ, second position F81, third position GTR + Γ. The nuclear locus (EF1-α) was analysed under the GTR + Γ model. Because of difficulty in amplifying nuclear DNA from older specimens, the EF1-α data set was substantially smaller than the COI data set and the two could not be combined and were therefore analysed as separate data sets.

For *Parapanteles*, the three genetic loci (COI, *Wgls* and ASpec) were analysed as a combined data set with each gene partitioned separately under the GTR + I + Γ model. As the problems of concatenation have been well documented (e.g. Degnan & Rosenberg, 2006), we conducted preliminary analyses of each gene separately to test for gene tree incongruence. The trees produced from each gene separately all showed a similar relationship between *Parapanteles* specimens (differing primarily in levels of branch support); thus, we combined the data for simplicity in the analyses described here.

In addition to the focused analysis of wasp specimens reared from *Eois*, we were interested in the distribution of *Eois* association within the larger *Parapanteles* genus. To estimate the phylogenetic relatedness of the *Parapanteles* reared from *Eois* in the context of the genus as a whole, COI sequences of *Eois*-associated wasps were analysed with other *Parapanteles* sequences from previous Costa Rican studies, which were downloaded from the BOLD database (Ratnasingham & Hebert, 2007). To simplify this analysis, *Eois*-associated *Parapanteles* specimens were included if they were either genetically or ecologically distinct (i.e. COI sequences were unique by at least one base pair that was reliably scored in forward and reverse sequence reads, or specimens were reared on caterpillars with different morphospecies names). These sequences were aligned using the Opal plug-in for Mesquite version 2.74 (Wheeler & Kececioglu, 2007; Maddison & Maddison, 2010), and the alignment was used in a Bayesian analysis using MrBayes with the same best-fit model as described above (GTR + I + Γ).

Bayesian analyses of sequence data for *Eois* and *Parapanteles* included four independent runs with three heated chains and one cold chain in each run (*Piper* sequences were not reanalysed). The Markov chain Monte Carlo (MCMC) parameters were set for six million generations sampled every 100 generations (*Eois*) and one hundred million generations sampled every 1000 generations (*Parapanteles*). The burn-in period was removed after graphical determination of stationarity using Tracer v1.4.1 (Rambaut & Drummond, 2007).

Because the phylogenetic relationships between *Piper* species have been previously established, rather than reanalyse the data, we present a reduced version (many of the outgroups and duplicate taxa removed) of the larger *Piper* phylogeny based on ITS1, ITS2, 5.8S and the *psbJ-petA* intron from Jaramillo *et al.* (2008). Sequence data from Jaramillo *et al.* (2008) were used to test for codivergence between *Piper*, *Eois* and *Parapanteles* (see Test of cospeciation below).

Molecular dating analyses

Within the general context of potentially coevolving host–parasite lineages, the timing of diversification is of inherent interest for each lineage. The timing of diversification in *Piper* has recently been addressed (Smith *et al.*, 2008b), but many of the species relevant to our study were not included in that analysis. We estimate the timing of diversification in *Eois*-associated *Piper* species using a recently described fossil calibration point (Martinez, 2011).

For *Eois*, we analyse the timing of diversification by calibrating a molecular clock with a date of origin for the group that has been published by Strutzenberger & Fiedler (2011). We do not yet have a calibration point enabling a robust divergence time estimate for *Parapanteles*. Instead, we roughly estimated the timing of diversification of *Parapanteles* onto *Eois* by applying the generic arthropod COI molecular clock estimate of 2.3% sequence divergence between lineages per million years (Brower, 1994) to the *Parapanteles* COI sequences.

We estimated divergence dates in *Eois* from the COI data set and in *Piper* from the ITS data set using Bayesian MCMC searches and an averaging approach to rate smoothing in the program BEAST version 1.4.8 (Drummond & Rambaut, 2007). Although no *Eois* fossils are known, the age of the genus has been estimated using fossils of related Geometridae (Strutzenberger & Fiedler, 2011). We used the estimated age and 95% confidence intervals from Strutzenberger & Fiedler (2011) for Neotropical *Eois* to calculate divergence times in this group by constraining the age of the genus *Eois* to 30 million years

before present ± 5 million years. A *Piper* fossil belonging to the Schilleria group identified from the upper Cretaceous (Maastrichian) was used to calibrate the divergence time estimation among *Piper* species. A Yule process speciation prior for branching rates was implemented and the GTR + I + Γ was applied to each data set with base frequencies estimated during the analysis. An uncorrelated log-normal model was applied to estimate the relaxed molecular clock. The analyses were run using the default MCMC parameters with the MCMC chains being set for 10 million generations and sampled every 1000 generations.

Character mapping and test of phylogenetic signal

To visualize the phylogenetic distribution of host use and parasitism across the three trophic levels, we mapped host use or parasitism onto each group's Bayesian consensus tree. Host associations were scored as a categorical, unordered trait at the generic level for *Parapanteles* and *Piper* (i.e. if a given wasp attacked any *Eois* species or a given *Piper* species was attacked by any *Eois*, a positive association was recorded, and if a wasp was not known to attack any *Eois* species or no record of *Eois* was associated with a given *Piper* species, a negative association was recorded) and mapped parsimoniously using Mesquite 2.74. Because parsimony analyses can sometimes produce misleading results (e.g. Goldberg & Igic, 2008), we also analysed the phylogenetic distribution of *Eois* host use using likelihood methods with Mesquite 2.74. On the *Eois* phylogeny, associations were scored as a categorical, unordered trait at the species level for both the COI and the EF1-α trees. To determine whether resource use contains significant phylogenetic signal or has evolved randomly across each phylogeny, we conducted permutation tests (Maddison & Slatkin, 1991) in Mesquite 2.74. We randomized the trait data onto the phylogeny 1000 times and again optimized the trait onto the tree to determine whether the observed phylogenetic distribution of the trait on the tree is statistically distinguishable from the null distribution.

Test of cospeciation

In order to test for evidence of cospeciation among the three trophic levels, pairwise tests were performed between each group (i.e. *Eois-Piper*, *Eois-Parapanteles* and *Piper-Parapanteles*). In contrast to methods that rely on tree topologies to test for cospeciation (e.g. treemap: Page, 1994), we employ a method that utilizes distance matrices based on DNA sequence data for each trophic level (Hommola *et al.*, 2009). These matrices were analysed for nonrandom associations between hosts and parasites using the permutation test of Hommola *et al.* (2009). This test uses distance matrices of two groups and a matrix of associations between these groups to estimate a correlation between distance matrices. This correlation is then compared to a null distribution of matrix correlations created through random permutations of the data. We used the Hommola *et al.* (2009) test because the method compares distances matrices rather than tree topologies and is therefore more robust to phylogenetic error and incomplete sampling than tree-based approaches (Legendre *et al.*, 2002; Hommola *et al.*, 2009). Nevertheless, the cryptic diversity and extreme richness of our focal groups suggest that incomplete taxon sampling remains a challenge for work on *Eois* and *Parapanteles*.

Results

Phylogenetic relationships

Eois

The final alignment for COI included all 94 *Eois* specimens and encompassed 556 base pairs. The final alignment for EF1-α included 51 specimens and encompassed 452 base pairs. The Bayesian analyses implemented in MrBayes and BEAST resulted in phylogenies with some well-supported nodes (Figs 1 and 2). Combining the COI and EF1-α data sets into a single, concatenated data set did not increase posterior probabilities for most nodes; therefore, we present the phylogenies as two distinct analyses. The parsimony and likelihood character reconstruction analyses were qualitatively identical but, for ease of visualization, we present the results of the parsimony analysis only (Fig. 1). Mapping host use on the EF1-α and the COI phylogenies shows a considerable amount of host conservatism among closely related individuals (Fig. 1). These radiations of *Eois* taxa (species and morphospecies) on the same host plant species are often supported by high posterior probabilities (Fig. 1). The randomization procedure testing the distribution of host use on the *Eois* phylogeny showed that host use was nonrandomly distributed across the tree (35 steps, $P = 0.001$).

Piper

A total of 52 plant specimens and 1715 bp were included in the phylogeny depicting relationships between *Piper* species (Fig. 2). In order to visualize the broader pattern of herbivory, we marked those *Piper* species that are known *Eois* hosts (Dyer *et al.*, 2008; Dyer *et al.*, 2011) on a previously published phylogeny containing additional species (Fig. S1; Jaramillo *et al.*, 2008).

Parapanteles

A total of 38 *Parapanteles* specimens and 2280 bp were included in phylogenetic analyses. The results of the Bayesian analysis estimating the diversity of *Parapanteles* suggested that six distinct clades (likely representing biological species, but possibly containing unresolved cryptic species) exist among *Eois*-attacking parasitoids (Fig. 3a). The Bayesian analysis exploring the

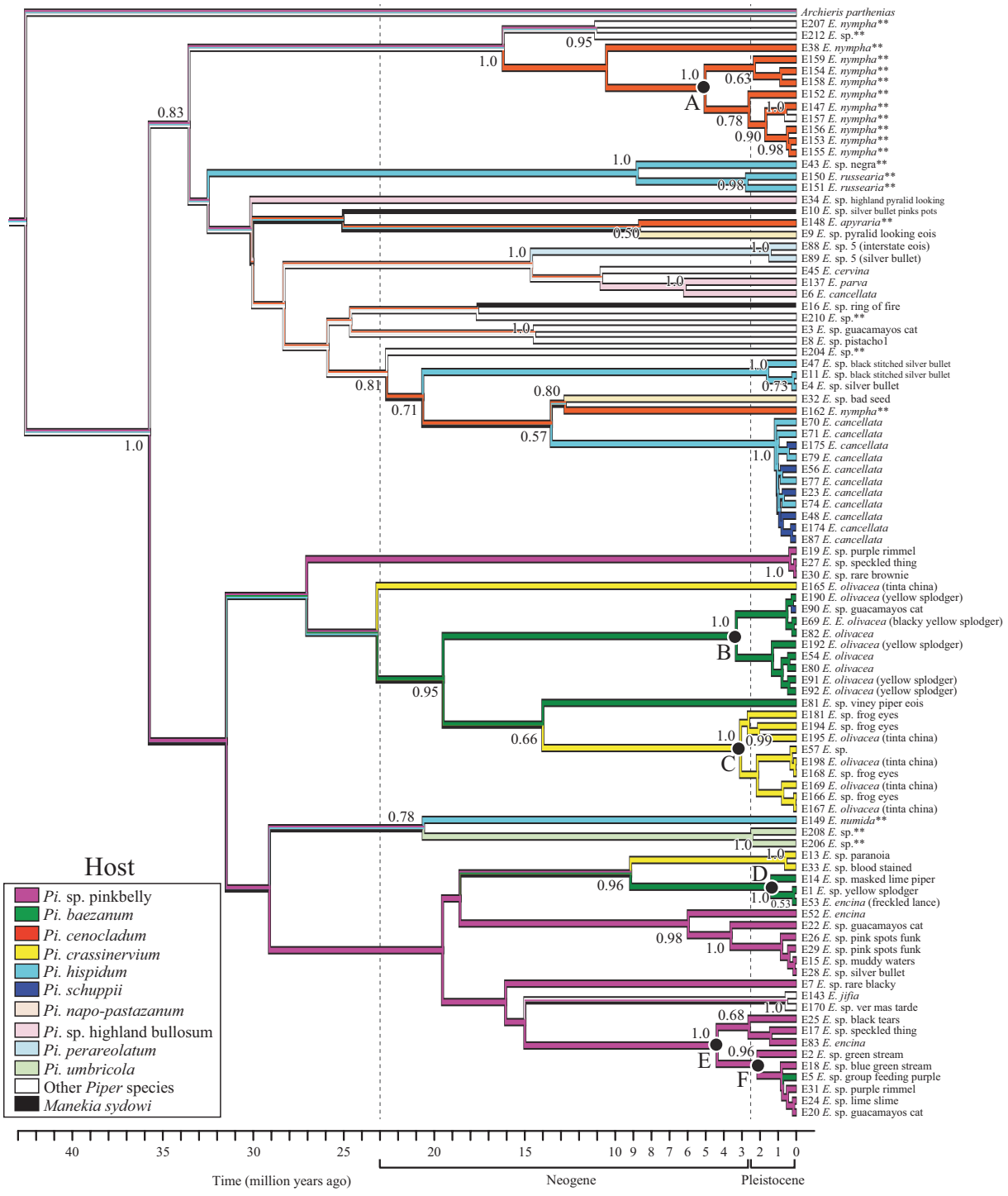

Fig. 1 Bayesian consensus tree and divergence dates of *Eois* based on COI estimated from BEAST. Posterior probabilities are given for nodes supported with values of 0.50 or above. Clades marked with black dots and labelled with A-F denote specific groups discussed in text. Branch colours indicate host plant affiliations based on parsimony reconstructions. Divergence times are indicated, and the Neogene and Pleistocene boundaries are marked. Terminal taxa names contain the voucher number and the species name (italicized) or morphospecies name (not italicized). Some taxa were identified based on adult morphology but had distinct larval morphology, in these cases the species name is given followed by the morphospecies name in parentheses. Taxa marked with ** were collected in Costa Rica, all others were collected in Ecuador (see Table S1 for details).

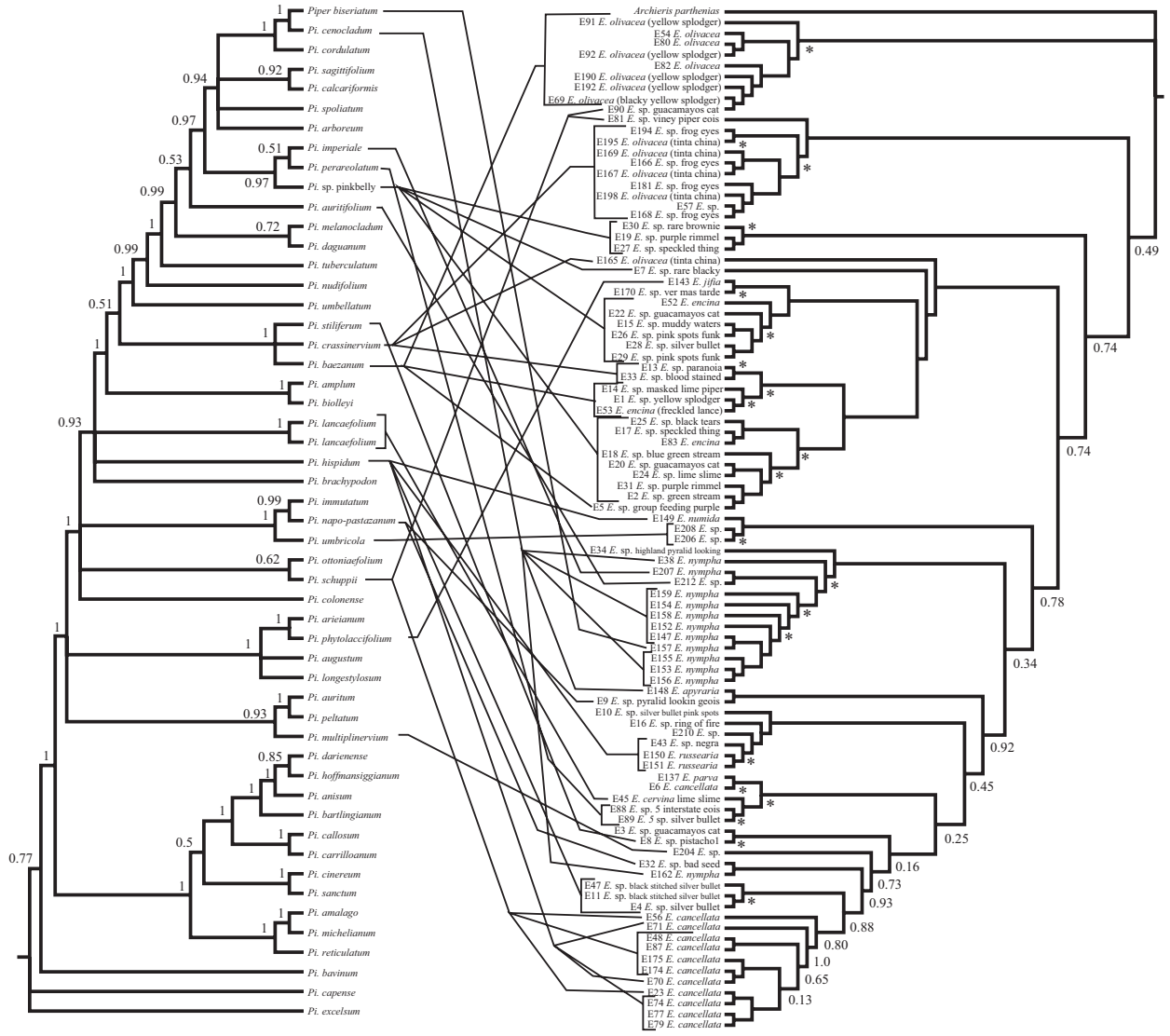

Fig. 2 Comparison of Bayesian consensus trees of *Piper* (ITS and *psbJ-petA*) and *Eois* (COI) estimated using MrBayes, and showing the known ecological associations between host and herbivore (lines connecting taxa). Because the phylogenetic relationships between *Piper* species have been previously established, we present a reduced version of the larger *Piper* phylogeny based on ITS1, ITS2, 5.8S and the *psbJ-petA* intron from Jaramillo *et al.* (2008). Phylogenetic analysis of *Piper* was performed using MrBayes version 3.1.2 as above but with a subset of the Jaramillo *et al.* (2008) data set that included all the *Eois* hosts and their pertinent sister groups (Table S2). The Bayesian analyses included four independent runs with three heated chains and one cold chain in each run with the HKY + Γ model applied to the combined data set. The Markov chain Monte Carlo (MCMC) parameters were set for ten million generations and sampled every 1000 generations. The first five million generations were discarded prior to the formation of a consensus tree (Jaramillo *et al.*, 2008). Posterior probabilities are reported for *Piper*, and posterior probabilities are reported for the backbone of *Eois*. Internal nodes marked with * on the *Eois* phylogeny indicate posterior probabilities of 0.95 or above. Lines connect plants that host *Eois* (see Table S2 for details).

relationship of *Parapanteles* that were reared from *Eois* within the broader context of the genus ($n = 47$) shows some phylogenetic structuring, with two small radiations of parasitoids specializing on *Eois* (Fig. 3b), among other clades specializing upon other host groups. Although host use was nonrandomly distributed across the phylogeny when viewed at the generic level (i.e. *Parapanteles* that attack *Eois*; 3 steps, $P = 0.001$), host use does not appear to be conserved at the species level. In other words, we found no evidence of radiations of *Parapanteles* onto single *Eois* species, in contrast to the pattern of multiple *Eois* species on single *Piper* host species.

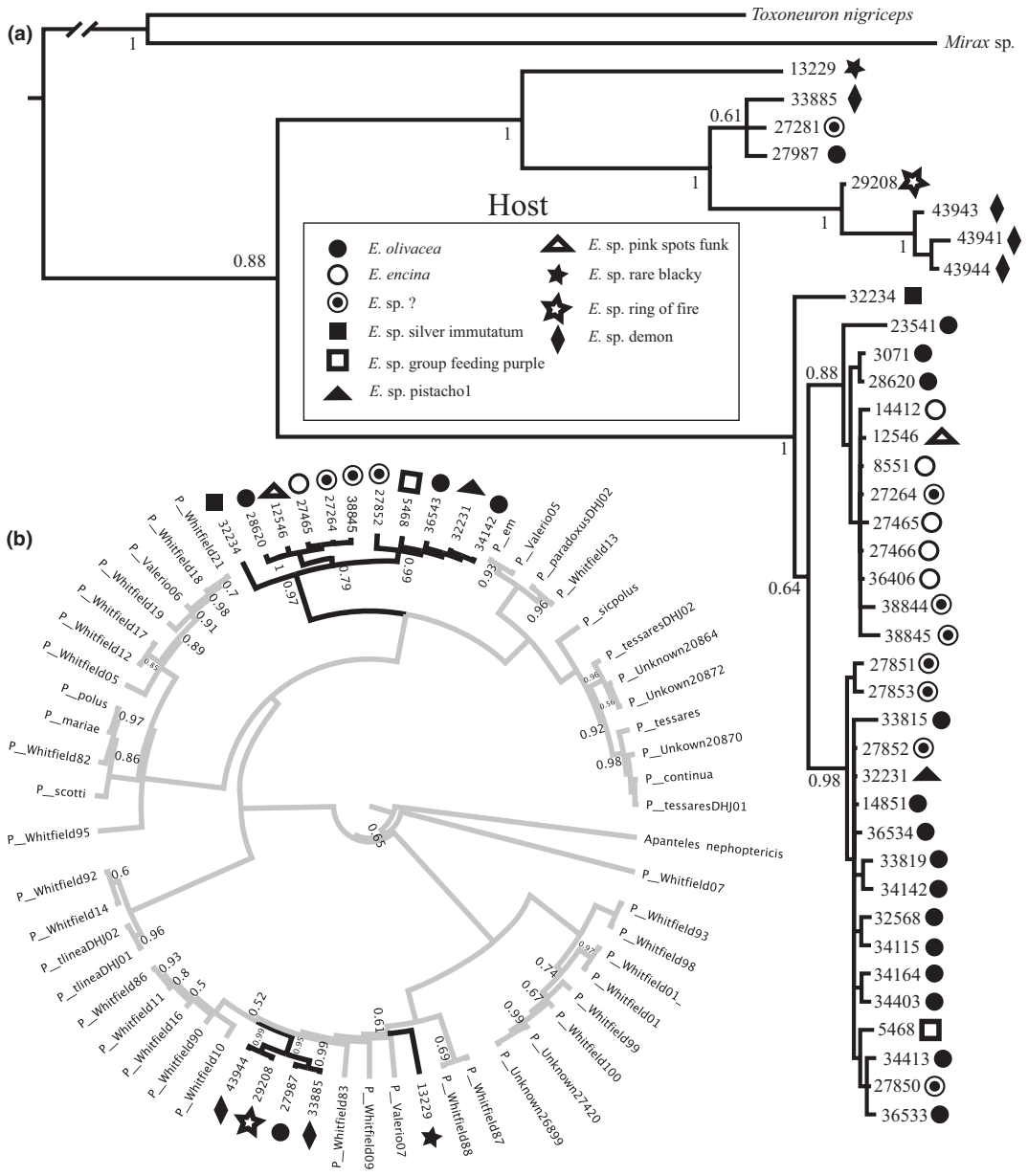

Fig. 3 (a) Bayesian consensus tree of *Parapanteles* wasps based on the combined data set (COI, *Wgls* and *ASpec*) for all specimens studied estimated using MrBayes. Posterior probabilities are given for nodes supported by a posterior probability of 0.5 or greater. (b) Bayesian consensus tree of the larger *Parapanteles* data set (COI only) estimated using MrBayes. Posterior probabilities are reported for nodes supported by 0.5 or greater. Taxa marked with black branches are known to parasitize *Eois*, taxa marked with grey are either not known to parasitize *Eois*, or the host associations are unknown. Terminal taxa are marked with voucher numbers for those specimens reared from *Eois* (see Table S3) or with species identifiers associated with the BOLD database. Symbols marking *Eois* species on the *Parapanteles* tree indicate the specific *Eois* morphospecies or species from which the parasitic wasp was reared.

Comparison with previous phylogenies

A phylogenetic hypothesis for a portion of *Eois* was recently published by Strutzenberger *et al.* (2010), which differs from our work in a number of ways. First, because *Eois* is so diverse and beta diversity is likely very high (Brehm *et al.*, 2003), our taxon sampling does not broadly overlap with Strutzenberger *et al.*'s (2010) phylogeny. Furthermore, their analyses focused on family- and genus-level host associations (Strutzenberger *et al.*, 2010), whereas our focus is on host associations with *Piper* at the species level. Thus, we investigated

resource use at a finer scale. Finally, rather than removing taxa that were genetically similar (< 2% genetic sequence divergence), we included all individuals that were genetically, morphologically or ecologically distinct considering both larval and adult stages. This approach is key to our study because the taxonomy of *Eois* remains uncertain, but more importantly because we were interested in the origins of diversity across multiple timescales, starting with the lowest level of diversification.

Molecular dating analysis

Our molecular dating analysis of *Piper* agreed with the finding of Smith *et al.* (2008b), showing that the genus underwent major diversification in the Neogene (Fig. S2). Based on the criteria for taxon inclusion explained above, the molecular dating analysis of *Eois* suggests several of the diversification events representing radiations of *Eois* onto single *Piper* hosts occurred during the Pleistocene (approximately 2.6–0.01 Ma: Fig. 1, clades A–F, Fig S3). Alternatively, if we apply the commonly used genetic distance of 2% divergence in COI to designate species, all speciation events occur before the Pleistocene. This illustrates the utility of our taxon selection criteria for investigating diversification at the lowest levels (which often includes morphologically and ecologically distinct taxa, discussed below), and the complexity of the continuum of diversification. Many of the deeper diversification events showing ancestral *Eois* species diverging and colonizing different *Piper* species occurred during the Neogene (approximately 23–2.6 Ma: Fig. 1). Our analysis of *Parapanteles* suggested that the colonization of *Eois* and the subsequent diversification of the parasitoids began between 1 and 1.25 Ma.

Test of cospeciation

Cospeciation tests were conducted between each of the three ecologically associated trophic levels in this system to determine whether divergence at one level is linked to divergence at a second level. The test of cospeciation between *Eois* and *Piper* suggested a high degree of correlation between phylogenies ($r = 0.35$, $P < 0.001$). As a complementary analysis, we conducted the test with a reduced data set that included only a single *Eois* specimen to represent each of the clades where multiple taxa feed on the same *Piper* host. This second analysis showed far less correlation between *Eois* and *Piper* phylogenies ($r = 0.09$, $P = 0.08$), indicating that correlation in the full data sets is due to high levels of host conservatism (i.e. radiations of closely related *Eois* species onto the same hosts) rather than to parallel cladogenesis or cospeciation. We did not find a significant correlation between the phylogenetic patterns of *Eois* and *Parapanteles* ($r = -0.03$, $P = 0.44$), or between *Parapanteles* and *Piper* ($r = 0.08$, $P = 0.22$). Although a test of cospeciation for tri-trophic systems has been developed (Mramba, 2010), we did not apply this method because, with the exception of the first comparison between *Piper* and the full *Eois* data set, pairwise tests among groups were not significant. Although these tests are based on sampling that only represents a portion of the overall diversity in these complex groups, these results are based on genetic distances rather than tree topologies, so they are not inherently biased by limited sampling (i.e. genetic distances remain relatively constant even when additional taxa are included).

Discussion

Phylogenetic patterns of host use

We have examined the hypothesis that diversification in one ecological partner is correlated to diversification in its associate by examining phylogenetic patterns of diversity, resource use and codivergence across three trophic levels in two Neotropical forests. Although ecological associations are generally nonrandomly distributed on the phylogenies we report, little evidence exists for cospeciation in these groups. Instead, host use clusters strongly on the *Eois* phylogenies, with multiple sister taxa attacking the same host plant species (Fig. 1). In this sense, many nominal *Piper* hosts seem to support their own mini-radiations of low-level diversification. In comparison, the distribution of *Eois*-attack across the *Piper* phylogeny is relatively dispersed, suggesting that all major groups of Neotropical *Piper* could potentially support *Eois* caterpillars (Fig. S1). Indeed, it is likely that *Piper* and *Eois* each host higher herbivore and parasitoid diversity than is reported here and as additional *Piper* species are examined for the presence of herbivores, records of *Eois* herbivory are expected to continue to accumulate. For example, an intensive study at La Selva (Costa Rica) has shown that all *Piper* species present at that site host at least one species of *Eois* (Dyer *et al.*, 2008). Thus, an apparent absence of herbivores on the *Piper* phylogeny is likely an artefact of sampling. It should be noted, however, that the addition of herbivory data would not change our result of host conservatism associated with several small *Eois* radiations from the same geographic area.

Host use is nonrandomly distributed on the *Parapanteles* phylogeny, where we see at least two small wasp radiations onto *Eois* (Fig. 3). For the distribution of host use on both the *Eois* and *Parapanteles* phylogenies, it is important to note that other patterns could have been observed. For example, it could have been the case that *Eois* sister taxa were more often associated with different *Piper* species, a scenario consistent with pervasive host-switching as a mode of speciation (Winkler & Mitter, 2008). For *Parapanteles*, an alternate possibility would have been dispersed relationships between species attacking *Eois*, such that the *Eois* specialists would have

appeared in many places across the wasp phylogeny (Fig. 3b).

Although host use displays some significant phylogenetic signal in both *Eois* and *Parapanteles*, we did not find evidence for parallel diversification or cospeciation. This might have resulted if, for example, *Eois* and *Piper* diversified in concert, or if *Eois* diversified onto *Piper* after the plants had radiated with sequential *Eois* diversification onto closely related (and possibly chemically similar) host clades. Our analysis of cospeciation between *Eois* and *Piper* did show a statistically significant correlation between the two phylogenies, which was driven by the presence of several diverse *Eois* clades all using the same host, as mentioned above. No evidence of cospeciation was found between *Parapanteles* and *Eois*, or between *Parapanteles* and *Piper*; the latter possibility, an association between parasitoid wasps and the plants harbouring their hosts, has been reported in other systems (Lopez-Vaamonde *et al.*, 2005).

There are, however, several examples of host plant shifts that lead to closely related *Eois* species feeding on closely related species of *Piper*. For example, there are two cases where a clade of *Eois* associated with *Piper crassinervium* (Clade C, Fig. 1) is sister to a clade of *Eois* associated with *Piper baezanum* (Clade B, Fig. 1), which is noteworthy because these two *Piper* species are close relatives (Fig. 2, Jaramillo *et al.*, 2008). These instances of closely related *Eois* feeding on closely related *Piper* might be due to phytochemical similarities between related plants, rather than an indication of shared evolutionary histories or strict cospeciation (Becerra, 1997). Nevertheless, if closely related hosts are ecologically, functionally or chemically similar due to descent, then *Eois* adapted to one of those *Piper* species should be predisposed to exploiting other related *Piper* lineages, contributing to the phylogenetic signal in the ecological associations.

Patterns of diversification

In tropical lepidopteran communities, larval morphology, including colour pattern, can be a more revealing indicator of species-level diversity than adult morphology (e.g. Hebert *et al.*, 2004). Consistent with this, we found that using variation in larval colour pattern to differentiate taxa was not always congruent with using a benchmark genetic distance (usually 2%) in COI for identifying species units (see Forister *et al.*, 2008 for a review of problems associated with the use of single marker 'barcoding' at low taxonomic levels). For example, we found a wide variety of larval patterns (Fig. 4), and even variation in life history strategies, within a group of specimens that are all closely related (< 2% COI sequence divergence: Clade E, Fig. 1). Although some of the colour pattern variation observed among closely related individuals might represent polymorphism within biological species, it is clear that at least some distinct species are present within clades separated by very small genetic distances. One extreme example appears in Clade E (Fig. 1), specifically regarding specimens E5 and E24. These specimens share nearly identical COI haplotypes (0.02% sequence divergence), yet are quite different morphologically and behaviourally, and feed on different host plants. Specimen E5 has the morphospecies name 'group feeding purple', which, as the name indicates, has gregarious larvae that are purple in colour (Fig. 4c). The specimen E31 (purple rimmel), however, has solitary larvae that are green and black (Fig. 4b). Furthermore, the adults of these specimens are morphologically distinct (Fig. 4b,c). Additionally, the nuclear gene EF1-α, typically far less variable COI (Reed & Sperling, 1999; Wahlberg *et al.*, 2005; Strutzenberger *et al.*, 2010), appears to support the possibility that members of this clade are genetically distinct (Fig. S4).

In addition to several radiations of genetically similar species that are morphologically distinct, our phylogeny indicates that several cryptic species likely exist in *Eois*. For example, *Eois olivacea* appears in multiple separate clades on the tree, indicating that some specimens, although morphologically similar, are distantly related. Another suggestion of cryptic diversity can be seen in Clade A (Fig. 1), which is composed entirely of specimens identified as *Eois nympha* because of morphological similarity (Fig. S5), yet the genetic diversity in this Clade A is equal to the diversity in clades made up of multiple morphologically distinct species (e.g. Clade E), further suggesting the existence of cryptic diversity.

Timing of diversification

Based on ancestral character state reconstructions and molecular dating analyses, the majority of host shift events in *Eois* are associated with older nodes, primarily Neogene divergence (Fig. 1). *Piper* also experienced a major diversification during the Neogene (Fig. S2; Smith *et al.*, 2008b), possibly due to the massive uplift of the Andes at that time (Gregory-Wodzicki, 2000). The diversification of *Piper* likely influenced the diversification of *Eois* with host shifts following the evolution of new *Piper* species. For example, the evolution of *Pi. crassinervium* and *Pi. baezanum*, which occurred between 22 and 5 Ma (Fig. S2), is temporally associated with Neogene host shifts and divergence in *Eois*, where ancestral species diverged onto those two host plants at around that same time (Figs 1 and S3; nodes connecting green and yellow branches).

In addition to Neogene host shifts and diversification, *Eois* also experienced several diversification events during the Pleistocene (Figs 1 and S3). These diversification events are unique in that they represent genetic divergence largely without host shifts, as described above. The diversification of *Parapanteles* onto *Eois* also occurred during the Pleistocene and may have been

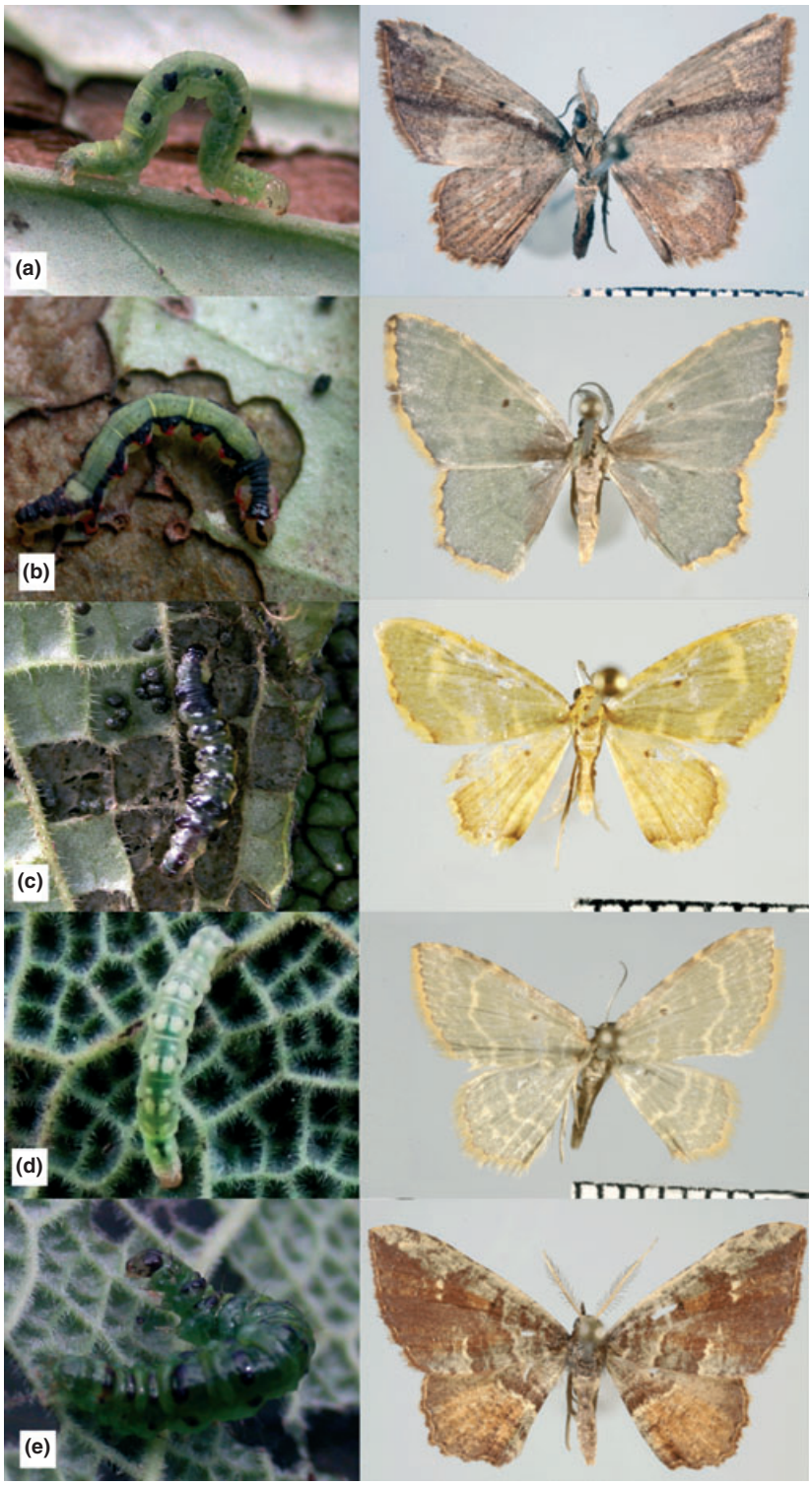

Fig. 4 Examples of the morphological diversity observed in closely related *Eois* specimens from clades D and E (Fig. 1). (a–c) represent individuals from Clade E and (d–e) represent individuals from Clade D. Images display the larval stage (3rd instar) on the left and the adult stage on the right for each individual. (a) is representative of E17 (speckled thing), (b) is representative of E31 (purple rimmel), (c) is representative of E5 (group feeding purple), (d) is representative of E1 (yellow splodger) and (e) is representative of E53 (*Eois encina*, freckled lance).

driven in some part by the diversification of *Eois* during that time.

Hypothesized mechanisms of diversification

Our phylogenetic results suggest a complex history of host shifts and host conservatism in *Eois*. These patterns of host use raise several questions that are not directly testable at this time, but which we present as hypotheses that could be useful for future work in this and other plant–herbivore–parasitoid systems. Although host shifts contribute to diversification in some groups (e.g. Fordyce, 2010), recent studies of herbivorous insects have also documented diversification without host shifts, finding instead that diversification was driven by geographic isolation (Imada *et al.*, 2011). Recent diversification events in *Eois* are unusual, however, because we observe several instances of diversification without host shifts occurring in the same general geographic location (clades A–E, Fig. 1). Pleistocene cooling led to a combination of range shifts and isolation into forest refugia in many Neotropical taxa (e.g. Hooghiemstra & van der Hammen, 1998; Bush *et al.*, 2004; Colwell *et al.*, 2008; Valencia *et al.*, 2010; but see Rull, 2011). Our results are consistent with the notion that Pleistocene climate cycles have had a dramatic effect on tropical diversity, and we hypothesize that such events have been influential in driving *Eois* diversification by isolating populations of hosts and conspecifics.

Pleistocene isolation does not, however, provide a mechanism in which genetic differentiation between *Eois* populations would be maintained after the Holocene reestablishment of widespread forest. We suggest that the cause of genetic differentiation in *Eois*, and the maintenance of this genetic diversity, could be a combination of vicariance, ecological speciation and landscape heterogeneity. In many tropical landscapes, soil composition is a complex mosaic of different types (Sollins *et al.*, 1994; Bennett, 2010). Recent evidence suggests that soil type can be a major factor influencing phylogeographical patterns in some plants (Alvarez *et al.*, 2009). Furthermore, chemical defences in *Piper* are highly variable along natural and experimental gradients in soil quality or nutrient availability, even within species along elevational and latitudinal gradients (Dyer *et al.*, 2004a,b; Dyer & Letourneau, 2007). This change in chemistry has well-documented effects on *Piper* tri-trophic interactions, such as affecting development rates and pupal weights in *Eois*, enhancing parasitism by *Parapanteles* due to sequestered toxins disrupting the immune response of *Eois*, or altering direct effects of *Piper* on the broader arthropod community associated with *Piper* and *Eois* (Dyer *et al.*, 2003, 2004a,b; Fincher *et al.*, 2008; Richards *et al.*, 2010). Because *Eois* are highly host specific, with each species restricted to an average of about two species of *Piper* (Dyer *et al.*, 2008; Connahs *et al.*, 2009), the movement of host plants during the Pleistocene would likely lead to colonization of different elevations and soil types, and therefore result in associations with host plants (even within the same nominal host plant species) having different chemical profiles than the ancestral populations. This continual range movement and resulting phytochemical shifts could drive local adaptation in *Eois* to host plants growing on specific soil types. This association of *Eois* populations on specific soils could persist even when widespread forests were reestablished in interglacial cycles.

Conclusions

Diversification in herbivorous insects is often attributed to the evolution of larval diet through host shifts and cospeciation or coevolution with host plants. Although strict cospeciation should not always be expected in herbivorous insects (Futuyma & Agrawal, 2009), coevolution can be expected to produce some level of congruence among phylogenetic histories of hosts and parasites. Our analysis of the species-rich moth genus *Eois* does not show evidence for parallel cladogenesis with its host plant, *Piper*, or with a parasitoid wasp, *Parapanteles*, that attacks it. In the case of *Parapanteles*, specialization onto *Eois* appears to have arisen relatively few times, and subsequent diversification on single host species is not evident. Instead of cospeciation as the primary driver of diversification in this system, we hypothesize that a combination of Neogene-aged host shifts and Pleistocene-aged radiations drove diversification in *Eois*; though, these processes may be too recent to exhibit a cascading impact on *Parapanteles*. In the Neogene, uplift of the Andes combined with diversification of *Piper* may have driven older diversification events in *Eois*. More recent diversification in *Eois* was possibly driven by Pleistocene climate changes, which created fragmented forest refugia and caused downslope migration of host plants. The glacial-aged movement of plants to new areas, specifically with new soil types, might have caused changes in the phytochemistry of the plants, driving local adaptation in *Eois*. This local adaptation is expected to have resulted in genetically distinct populations on specific soil types, which may have led to the recent diversification within this complex, tropical group.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Bayesian consensus trees of *Piper* from the combined dataset (ITS and *psbJ-petA*), redrawn from Jaramillo *et al.* (2008).

Figure S2 Bayesian consensus tree and divergence dates of *Piper* based on the ITS dataset estimated from BEAST.

Figure S3 Bayesian consensus tree and divergence dates of *Eois* based on COI estimated from BEAST.

Figure S4 (a) Bayesian consensus tree of *Eois* based on EF1-α estimated using MrBayes. Branch colors indicate host plant affiliations based on parsimony reconstructions. Tree is drawn as a cladogram for ease of visualization of host plant affiliations and posterior probabilities are reported at nodes. Terminal taxa names contain the voucher number and the species or morphospecies name (see Appendix 1 for details). (b) Bayesian consensus tree of *Eois* based on EF1-α with branch lengths to illustrate sequence divergence.

Figure S5 Examples of the morphological diversity observed in the cryptic species complex *E. nympha* from Clade A (Fig. 1).

Table S1 Detailed information about the *Eois* specimens used in this study including species identification numbers [corresponding to rearing numbers given in Gentry & Dyer (2002) and Dyer *et al.* (2011)], voucher numbers, host associations, collection localities, and GenBank accession numbers.

Table S2 Detailed information about the *Piper* specimens used in this study including species names, host associations, collection localities, voucher identification numbers, and GenBank accession numbers.

Table S3 Detailed information about the *Parapanteles* specimens used in this study including species identification numbers, host associations, plant associations, and GenBank accession numbers.

Table S4 Details of amplification and sequencing primers used in this study are indicated along with annealing temperatures and references.

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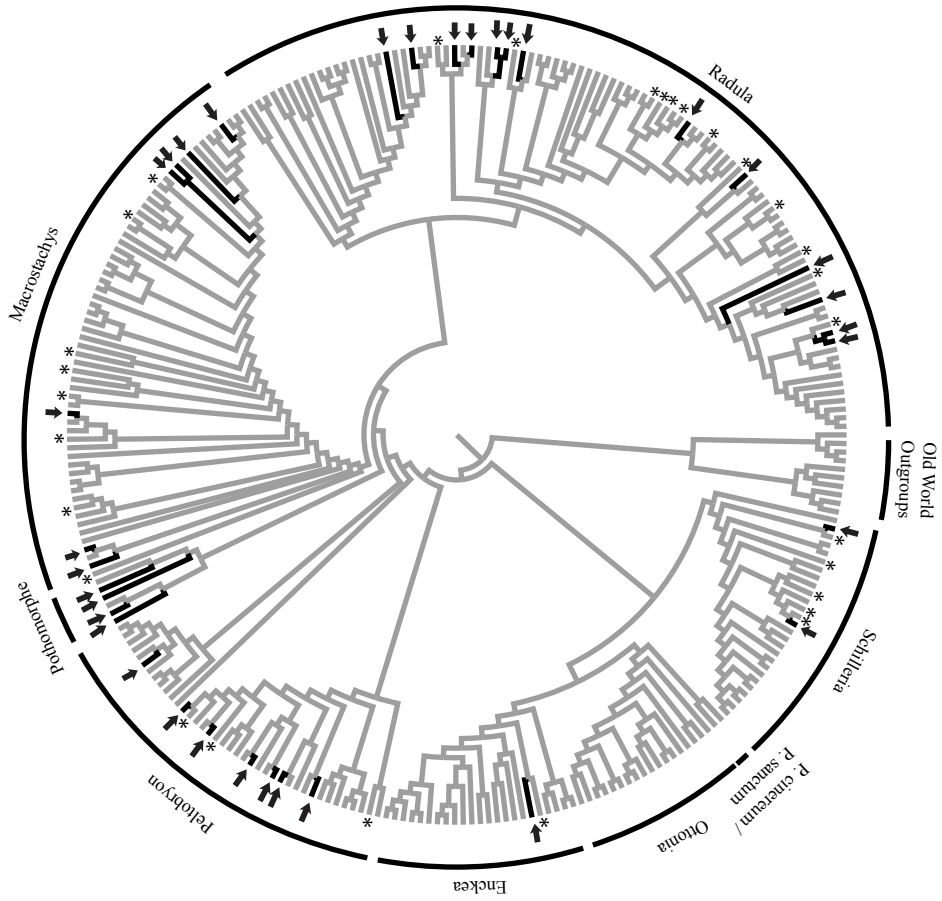

Figure S1. Bayesian consensus trees of Piper from the combined dataset (ITS and psbJ-petA), redrawn from Jaramillo et al. (2008). Clade and subclade names correspond to the descriptions given by Jaramillo et al. (2008). Piper species from which Eois have been reared (Dyer et al. 2011) are marked in black and indicated with an arrow, and those present at study sites, but not attacked are marked with an asterisk. This tree includes many Piper species not present at any of the study sites and, therefore, not surveyed for Eois. As a result, this figure is likely an underrepresentation of the proportion of Piper attacked by Eois. Furthermore, Eois is likely to be much more evenly distributed across Piper species than is suggested by this tree. For example, the distribution of section Ottonia is almost completely Brazilian and species of this section have yet to be surveyed for Eois. Similarly, many species of section Enckea are found in Mexico and northern Central America and only a few species are present at any of the study sites.

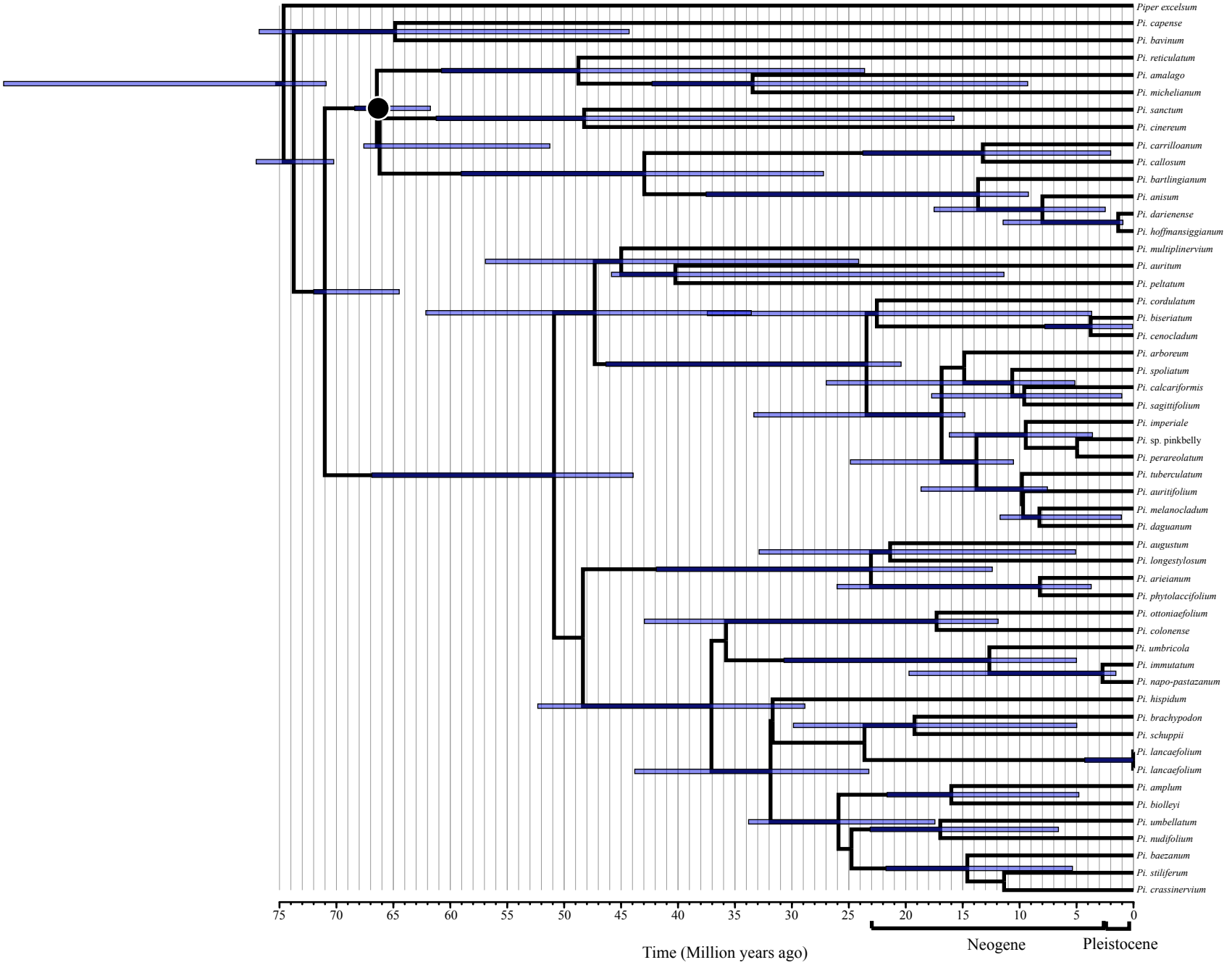

Figure S2. Bayesian consensus tree and divergence dates of *Piper* based on the ITS dataset estimated from BEAST. The node marked with a black circle marks the fossil calibration point. 95% credibility bars are given for nodes supported with a posterior probability >0.50.

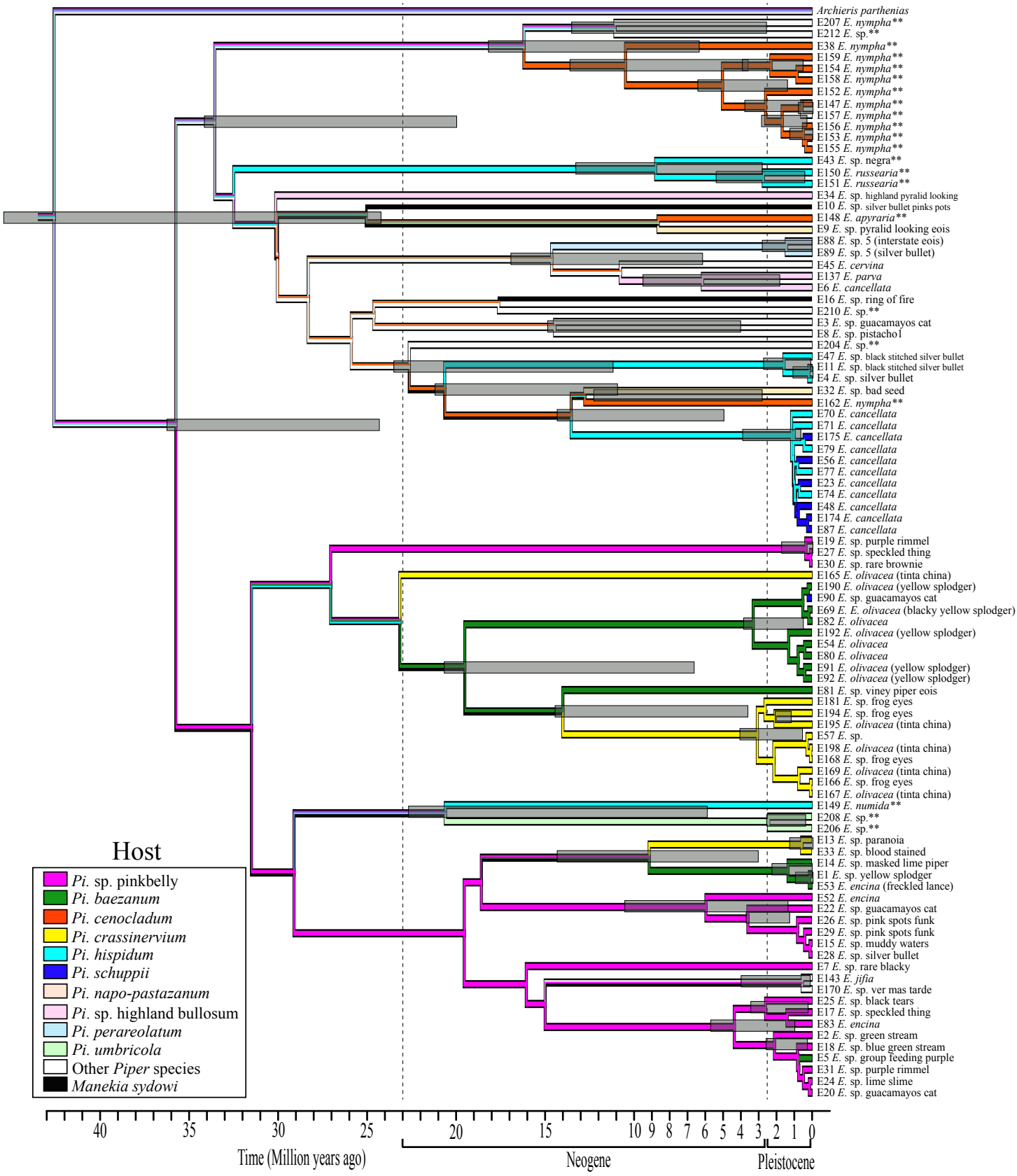

Figure S3. Bayesian consensus tree and divergence dates of *Eois* based on COI estimated from BEAST. 95% credibility bars are given for nodes supported with a posterior probability >0.50.

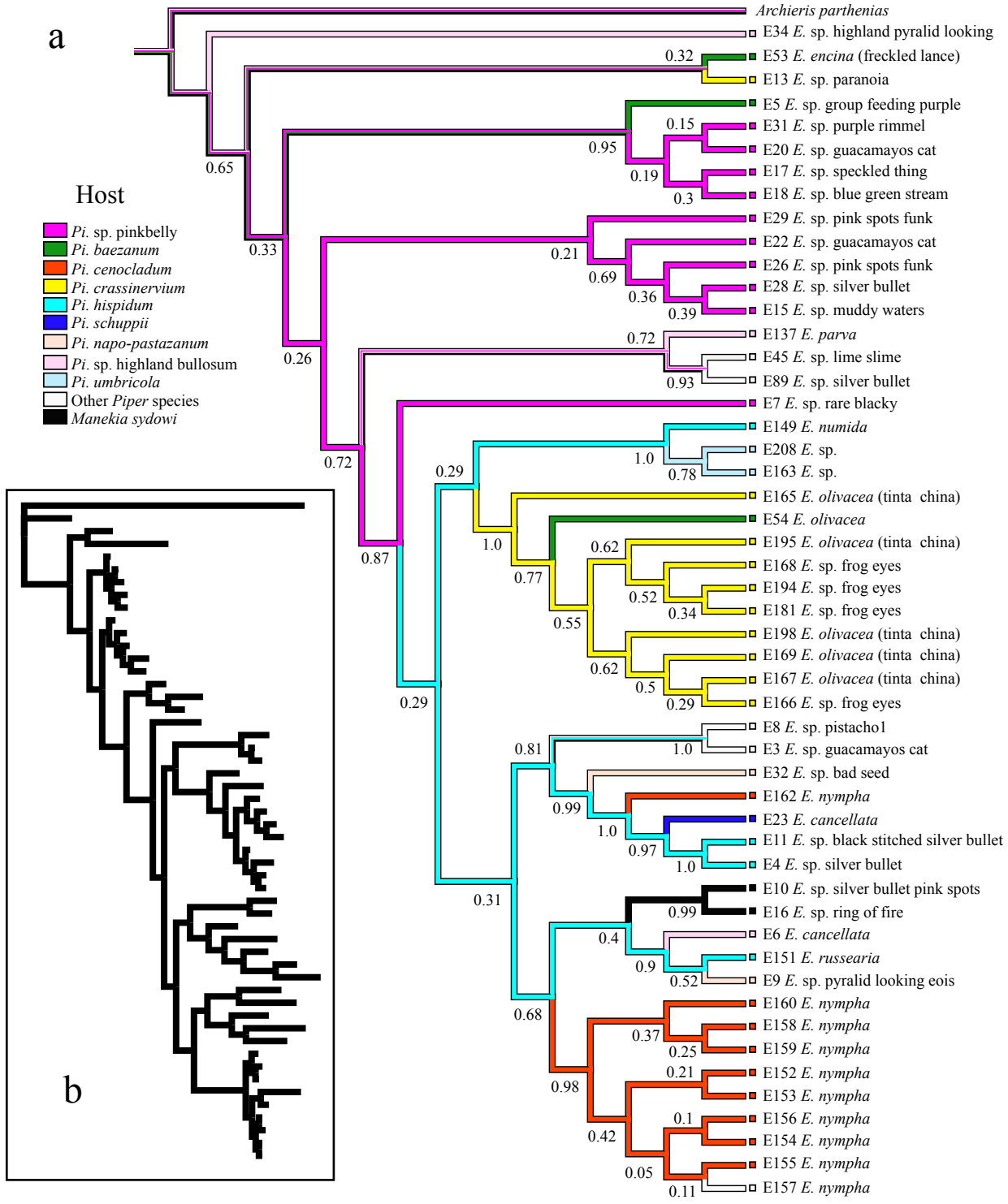

Figure S4. a) Bayesian consensus tree of *Eois* based on EF1- α estimated using MrBayes. Branch colors indicate host plant affiliations based on parsimony reconstructions. Tree is drawn as a cladogram for ease of visualization of host plant affiliations and posterior probabilities are reported at nodes. Terminal taxa names contain the voucher number and the species or morphospecies name (see Appendix 1 for details). b) Bayesian consensus tree of *Eois* based on EF1- α with branch lengths to illustrate sequence divergence.

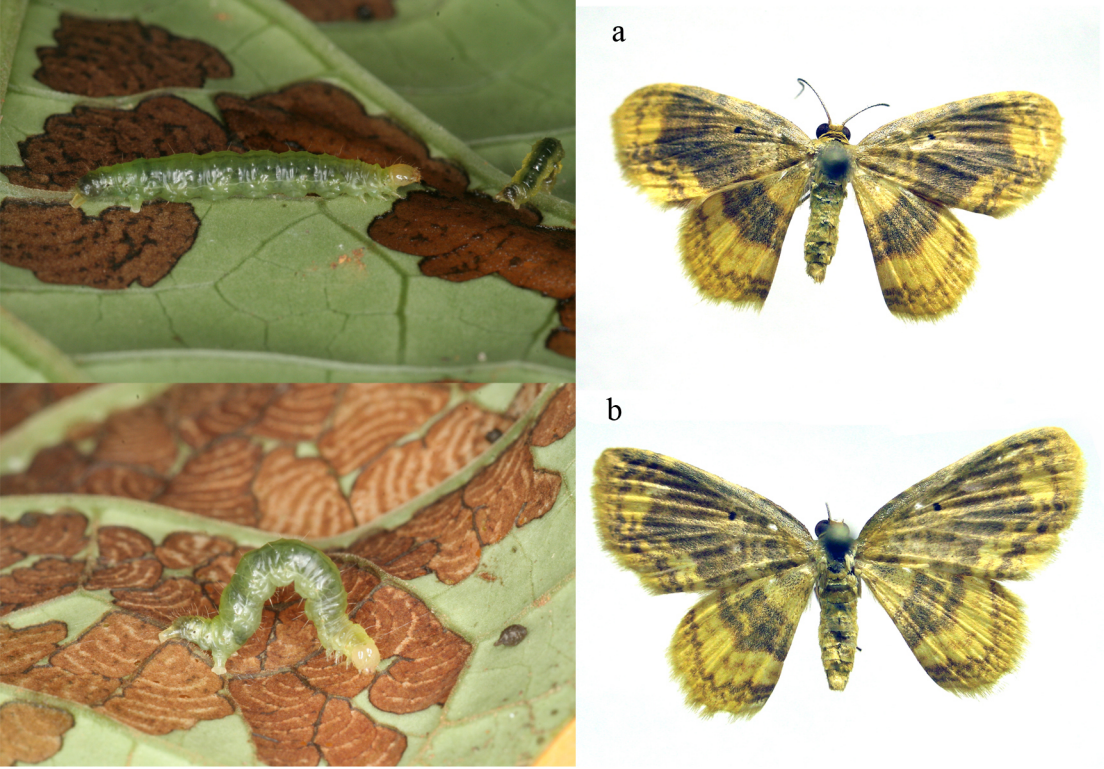

Figure S5. Examples of the morphological diversity observed in the cryptic species complex E. nympha from Clade A (Fig. 1). Because larvae look identical for all E. nympha specimens, images are not available for the exact specimens included in the phylogeny. Instead, larval pictures are included to give a representation of the similarity in larvae across the clade. Adult images show the morphological similarity of the genetically distinct specimens from Clade A. The specimen marked a) displays the dorsal wing pattern of E157 and the specimen marked b) displays the dorsal wing pattern of E159.