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Rapid diversification and not clade age explains high diversity in neotropical *Adelpha* butterflies

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Latitudinal gradients in species richness are among the most well-known biogeographic patterns in nature, and yet there remains much debate and little consensus over the ecological and evolutionary causes of these gradients. Here, we evaluated whether two prominent alternative hypotheses (namely differences in diversification rate or clade age) could account for the latitudinal diversity gradient in one of the most speciose neotropical butterfly genera (*Adelpha*) and its close relatives. We generated a multilocus phylogeny of a diverse group of butterflies in the containing tribe Limenitidini, which has both temperate and tropical representatives. Our results suggest there is no relationship between clade age and species richness that could account for the diversity gradient, but that instead it could be explained by a significantly higher diversification rate within the predominantly tropical genus *Adelpha*. An apparent early larval host-plant shift to Rubiaceae and other plant families suggests that the availability of new potential host plants probably contributed to an increase in diversification of *Adelpha* in the lowland Neotropics. Collectively, our results support the hypothesis that the equatorial peak in species richness observed within *Adelpha* is the result of increased diversification rate in the last 10–15 Myr rather than a function of clade age, perhaps reflecting adaptive divergence in response to the dramatic host-plant diversity found within neotropical ecosystems.

Keywords: divergence times; diversification rates; phylogeny; Neotropics; Limenitidini; species richness

1. INTRODUCTION

The latitudinal gradient of increasing species richness from the poles towards the equator is one of the most general and well-known biogeographic patterns [1–5]. This pattern holds true for both terrestrial and marine taxa, and shows no dependence on dispersal or thermoregulatory strategies [2]. Yet, despite agreement on the pattern, the mechanisms responsible are hotly debated, and there exist a multitude of likely interacting hypotheses [6–17].

One major proximate mechanism for the latitudinal diversity gradient is that of differential diversification rates between temperate and tropical regions, with higher rates of speciation and/or lower rates of extinction driving high tropical diversity [18]. Alternatively, differences in clade age, and not in diversification rates *per se*, may underlie variation in species richness among lineages [16,19,20]. The majority of studies to date have been based on comparisons across multiple clades or species, which are obviously important for increasing statistical power as well as testing the generality of mechanisms. However, studies of related clades that differ strikingly in diversity between temperate and tropical regions can also be informative, where knowledge about particular ecological traits can permit generation of more specific hypotheses. Here, we evaluated two alternative hypotheses for the origin of the latitudinal diversity gradient (namely differences in diversification rate or clade age) by reconstructing the phylogeny of a diverse group of butterflies—the tribe Limenitidini, which has both temperate and tropical representatives.

Our study focuses on the American Limenitidini, which contains two genera: the speciose, largely tropical *Adelpha*, and the much less diverse, temperate *Limenitis*. The genus *Adelpha*, commonly known as 'sisters', ranges from northwestern North America to Uruguay in South America [21]. Containing 89 described species and approximately 124 additional subspecies, it is one of the largest neotropical genera, with a peak in species richness at the base of the eastern Andes (figure 1), where local community diversity can be as high as approximately 40 species [21,22]. The genus displays remarkable diversity among larval stages in morphology, behaviour and host-plant specialization [23], with host-plant records for 44 species from at least 22 different plant families, including 66 genera and more than 116 different plant species [22]. By contrast, the most basal *Adelpha* clade, the montane *alala* group, resembles remaining Limenitidini genera in North America, the Palaearctic and temperate Asia in having

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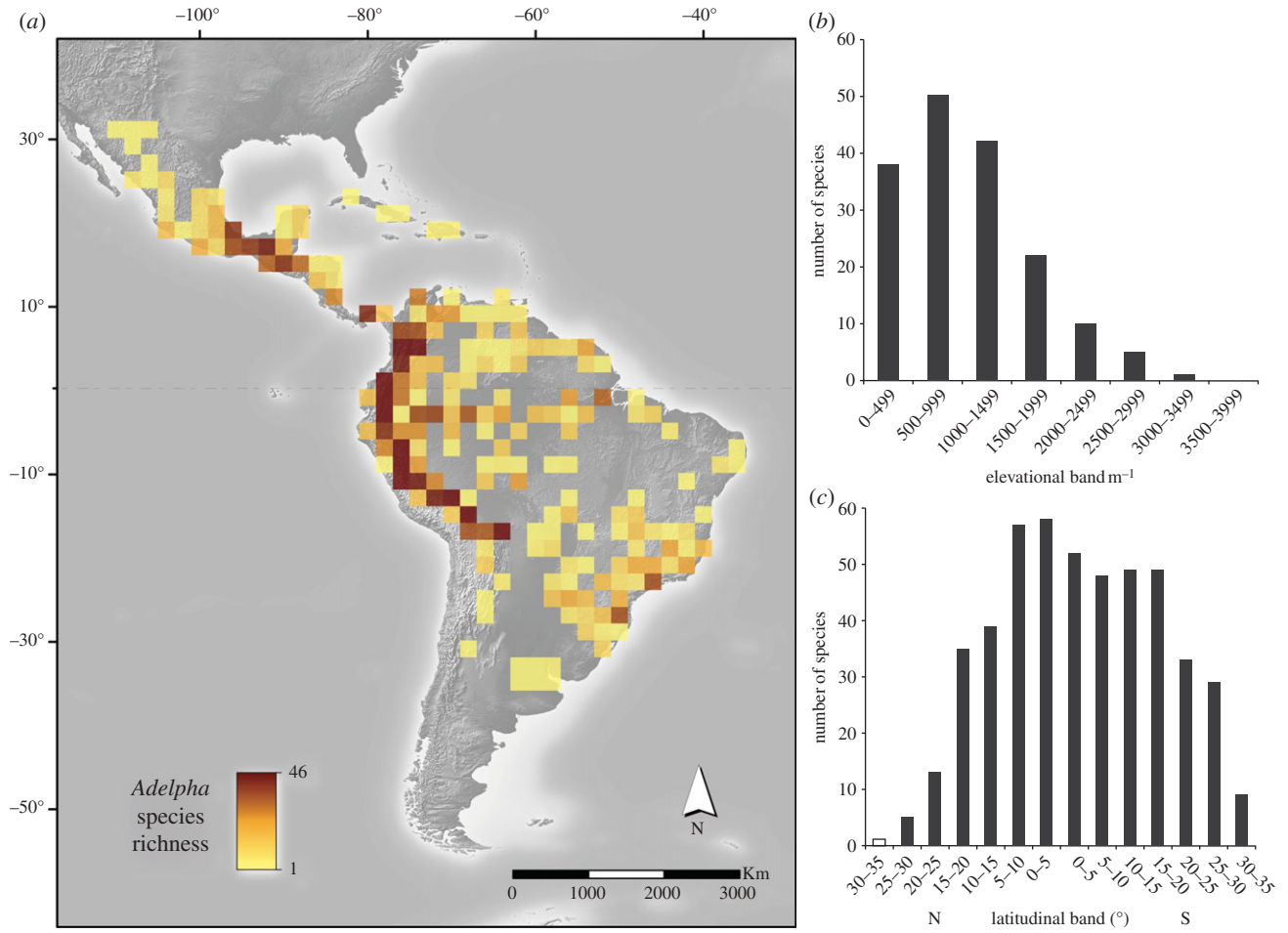

Figure 1. (*a*) Patterns of species richness in *Adelpha* butterflies across the neotropical region. Graphs illustrate patterns of (*b*) attenuating *Adelpha* species diversity with elevation above sea level along an equatorial East Andean elevational gradient and (*c*) increasing species diversity towards the equator.

only a few species confined to a relatively narrow host-plant range [24].

Based on a phylogeny inferred from morphology, Willmott [21] suggested that *Adelpha* are more closely related to certain Asian species than to the North American *Limenitis*, and that the disparity in diversity between the two American limenitidine genera probably represents, at least in part, the earlier arrival of *Adelpha* in the New World and consequent longer period of diversification. However, an alternative hypothesis is that a shift from temperate or montane habitats to tropical or lowland habitats allowed *Adelpha* access to a much greater range of host plants and resulted in accelerated diversification. Since phylogenetically informative morphological characters are remarkably few in the Limenitidini, especially for inferring relationships among genera, a well-supported phylogeny is needed to critically discriminate between these contrasting hypotheses. Here, we present a well-resolved molecular phylogeny of the tribe, including species-level sampling of the genus *Adelpha* and the temperate genus *Limenitis*. We test for evidence of whole-tree disparity in diversification rates, localize lineage-specific shifts in rates of diversification and estimate divergence times across the combined species-level phylogeny, and use these results to address two related questions. First, has the rate of diversification within *Adelpha* been more rapid than in temperate genera, or is the genus simply older than temperate groups with fewer species [21]? Second, how have biotic factors, such as biogeographic differences in host-plant richness, probably influenced the rate of diversification among temperate and tropical limenitidine genera?

2. MATERIAL AND METHODS

(a) *Taxon sampling*

Given the high diversity of species and wing-pattern races in *Adelpha*, and the large geographical range of the genus (figure 1), our sampling focused on species that represent apparent major clades based on morphology [21], and included 38 species and four subspecies for a total of 42 *Adelpha* taxa (electronic supplementary material). In addition, because knowledge of the relationship between *Adelpha* and the North American admirals (*Limenitis*) is relevant to understanding the biogeographic history of *Adelpha*, we sampled all of the Nearctic species of *Limenitis* as well as a large number of Palaearctic species. Finally, to place this radiation in a broader phylogenetic context, we included representative species from seven Limenitidini genera, three genera from the sister tribe Neptini and one representative of the tribe Parthenini (electronic supplementary material), resulting in a total of up to 72 taxa used for phylogenetic analyses.

(b) *Molecular methods*

Genomic DNA was extracted from muscle tissue and/or appendages using the commercial DNeasy Tissue Kit and

the standard protocol for animal tissue (Qiagen Corp., Valencia, CA, USA). Isolated DNA samples were quantified by spectrophotometry and diluted to concentrations of 50 ng μl^{-1}. For the complete set of 72 taxa (electronic supplementary material), we amplified three gene regions traditionally used in phylogenetic studies of the Lepidoptera: the cytochrome oxidase subunit I (*coxI*) of the mitochondrion, and two nuclear loci, elongation factor 1-α (*ef1-α*) and wingless (*wg*). Because we are also interested in examining the deeper parts of the tree and the more general patterns of diversification, we used a reduced taxon set of 29 species, and for each we amplified an additional three nuclear gene regions recently shown to provide good phylogenetic signal for among-clade relationships in other nymphalids [25]: glyceraldhyde-3-phosphate dehydrogenase (*gapdh*), isocitrate dehydrogenase (*idh*) and ribosomal protein S5 (*rps5*). Using both the 72 and 29 taxon datasets, we were thus able to identify within- and among-clade patterns of evolution.

(c) *Phylogenetic inference*

We employed both maximum-likelihood (ML) and Bayesian inference (BI) approaches to infer phylogenies for locus-specific and multilocus datasets. ML inferences were conducted with RAxML v. 7.2.6 [26,27] for locus-specific and combined partitioned mixed-model datasets using GTRCAT for the bootstrapping phase and GTRGAMMA for final tree inference, allowing the algorithm to estimate the proportion of invariant sites and optimize per-gene branch lengths. We analysed the aligned sequence datasets independently for phylogeny estimation of each gene region (*coxI*, *ef1-α*, *wg*, *gapdh*, *idh* and *rps5*), and by conducting tree searches under varying combinations of gene regions and partitioning strategies. Inferred support for nodes in the ML trees was first evaluated by letting the RAxML algorithm automatically determine the number of bootstrap replicates required for confidence, which varied from 100 to 500 iterations. Final bootstraps runs were performed with 1000 replicates.

BI was conducted with MrBayes v. 3.1 [28,29] for each of the sequence alignment datasets (as above), with substitution model settings estimated by MrModeltest v. 2.3 [30]. Gamma shape parameters, proportions of invariant sites, state frequencies and substitution rate matrices were unlinked, and rate parameters varied across gene and codon partitions. We based the final BI estimates for the individual gene tree and combined gene datasets from the results of two independent runs, each employing five coupled chains (one cold and four incrementally heated) for 10 million generations with a sampling frequency of 1000. We conservatively discarded the first 5000 sampled trees from each run as burn-in after inspection of convergence diagnostics, resulting in posterior probabilities taken from the remaining portion of sampled trees.

(d) *Analysis of diversification rates*

We tested the null hypothesis of equal diversification rates across the *Adelpha* phylogeny using a single-tree analysis of diversification rate variation implemented in SymmeTREE v. 1.1 [31,32]. Whole-tree diversification rate variation was estimated with two rate-shift statistics (M_{II} and M_{Σ}) and a tree imbalance statistic (B_1), over the whole *Adelpha* phylogeny [31,32]. The empirically generated distribution of species diversity across the *Adelpha* phylogeny was compared with the hypothesis that a shift in habitat (from temperate to tropical) and a consequent increase in potential host-plant diversity resulted in an overall change in the rate of diversification.

Null expectations of no rate variation were calculated under an equal-rates Markov random-branching model (ERM), assuming a continuous-time, discrete-state, pure-birth Markov process with a constant branching probability for each lineage tip through time. Under this model, the cumulative probability of realizing a diversity partition more extreme than the observed partition for sister groups is calculated for each individual tree node, and then generalized across the whole tree. The cumulative ERM probability of both the product (M_{II}) and sum (M_{Σ}) of all individual nodal probabilities is then compared with a null distribution derived by Monte Carlo simulation [32].

Likelihood ratio tests were used to assess the probability of a shift in diversification rate as extreme as that observed along a specific internal branch. One caveat is that although these explicitly model-based methods are robust to uncertainty associated with branch lengths/duration and can accommodate incompletely resolved trees, they are sensitive to taxon sampling. However, in the case of the present study, increased sampling of *Adelpha* is predicted to *increase* tree imbalance because the vast majority of the unsampled species occur in the lowland tropics clade. Thus, evidence for differential diversification is likely to be a conservative result.

(e) *Timing of divergences*

We tested temporal hypotheses of species diversification by estimating lineage divergence times using the Bayesian Markov chain Monto Carlo (MCMC) relaxed clock method implemented in the program BEAST v. 1.5.4 [33]. Lineage timing events were estimated with a combined sequence dataset for 29 taxa (containing *coxI*, *ef1-α*, *wg*, *gapdh*, *idh* and *rps5*), with each gene partition unlinked and modelled as in the MrBayes analyses. We used an uncorrelated lognormal tree prior, a birth–death prior on speciation and Jeffrey's priors on substitution model parameters. No fossil constraints were available for node calibration, but a recent study of the evolutionary history of Nymphalidae (the clade containing *Adelpha* and its closest relatives) reports several putative lineage divergence times based on host-plant age (maximum constraints) and butterfly fossils (minimum constraints [34]). Using the estimates of Wahlberg *et al.* [34], we constrained the root of the clade Limenitidini to have a mean age of 19 Myr (s.d. = 2.5 Myr), and enforced a uniform prior of 19–10 Myr on the age of the split between the lineages leading to *Adelpha bredowii* and *Limenitis reducta*.

3. RESULTS

(a) *Phylogenetic analyses*

The phylogenies inferred by ML and BI are generally similar in shape and support for individual gene and combined-gene datasets (figures 2 and 3; electronic supplementary material). Similarly, tree topologies of individual genes were similar to the phylogenies estimated with the differing combined gene region and partition datasets; however, each sequenced gene region alone had lower branch support and ambiguous relationships that conflicted with the combined gene datasets (see electronic supplementary material). The gene tree of the *wg* dataset

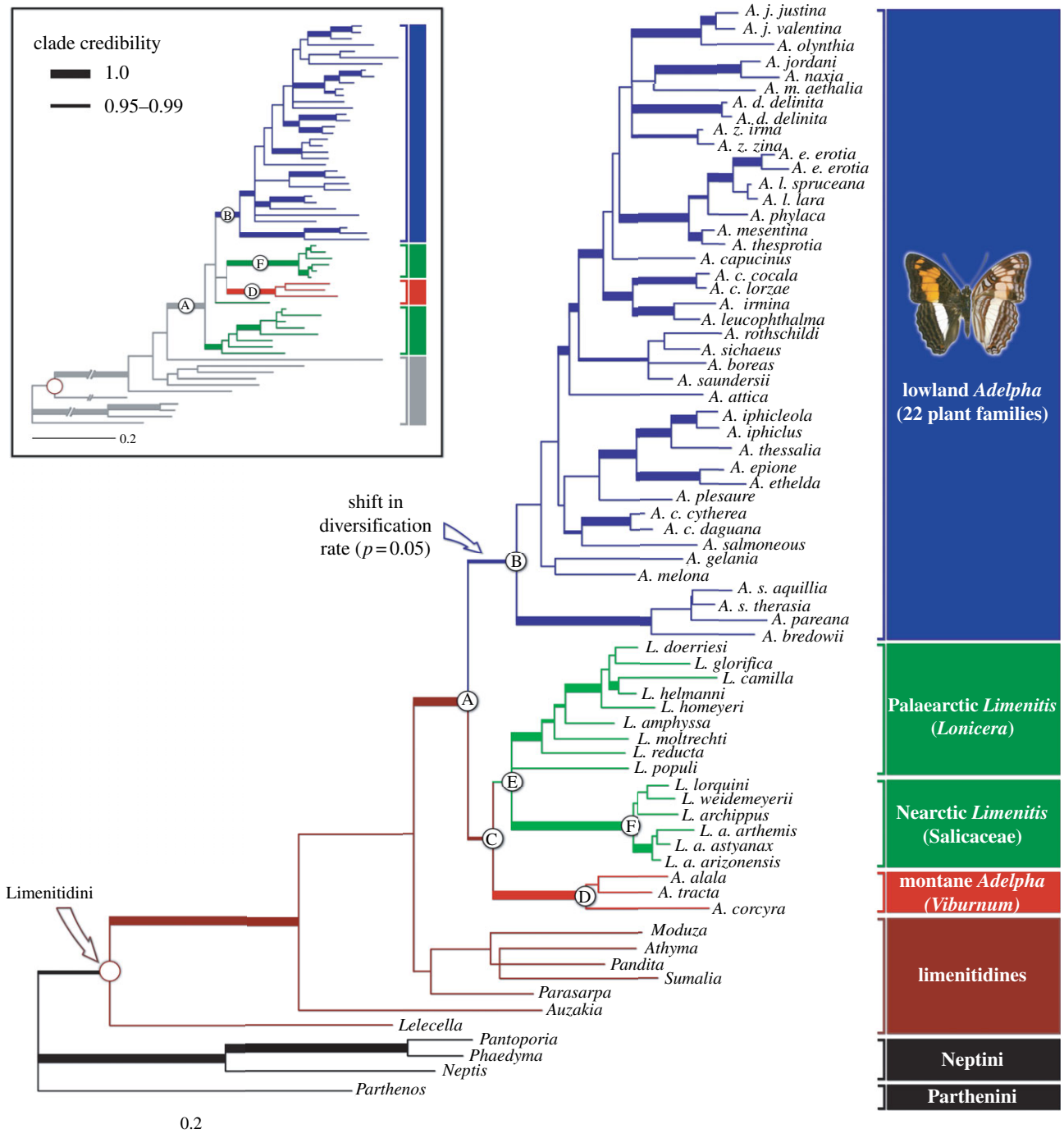

Figure 2. Bayesian consensus phylogram based on a mixed-model data partition (by gene and by codon position) of combined sequence data for *coxI* and *ef1-α*. The inset phylogram is based on dataset of all three gene and codon partitions. Branch weights represent posterior probabilities for clade support. The labelled nodes correspond with the inset tree and demonstrate the effect of *wg* on phylogenetic resolution. A shift in the species diversification rate occurred for the *Adelpha* clade containing all lowland species along the branch subtending from node A and leading the lineages contained by node B.

produced equivocal relationships at various depths across the tree and hence was the least informative gene region for resolving phylogenetic relationships, and even contrasted some of the relationships supported independently by the remaining gene sequence datasets. We therefore focused our analyses of the 72-taxon dataset on the results obtained with *coxI* and *ef1-α* sequence data, and, as shown in figure 2, the decaying effect of *wg* on portions of the phylogeny is quite apparent (evident in the figure inset, nodes C and E). The relationships among the Limenitidini genera used as outgroup taxa (excluding *Limenitis* and *Adelpha*) are unresolved, and this pattern appears across individual and combined gene tree analyses (electronic supplementary material; but see [34]).

The broader phylogenies from both datasets recover a lineage containing all *Adelpha* and *Limenitis* species supported by BI and ML bootstrap analyses (node A, figures 2 and 3). On closer inspection, this lineage contains both lowland and montane *Adelpha* lineages and, because of insufficient information to suggest otherwise, an unresolved assemblage of species named within the *Limenitis* genus. Analyses of both the 72- and 29-taxon

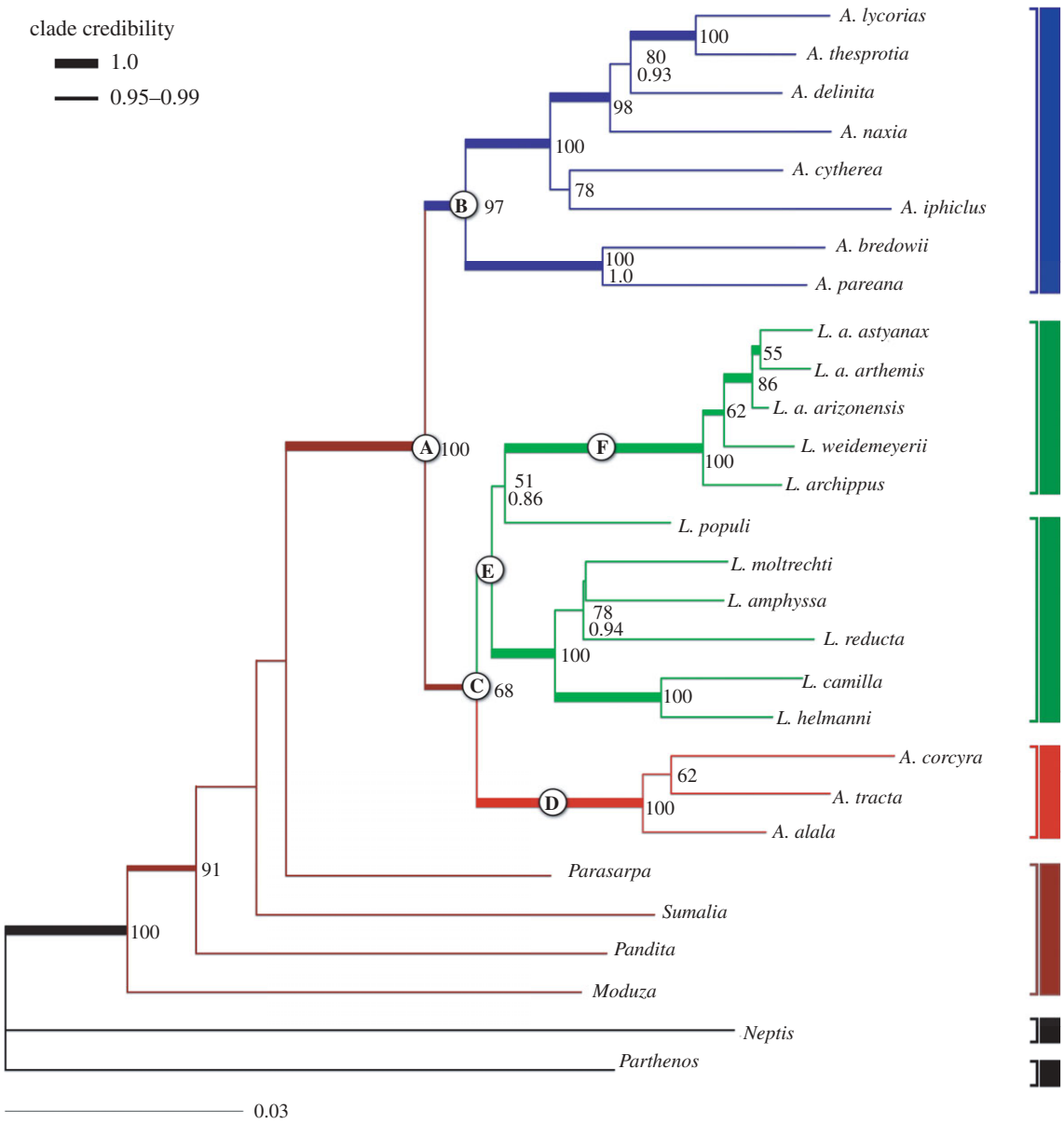

Figure 3. Bayesian consensus phylogram based on the combined dataset of six genes, partitioned by gene and codon for 29 taxa. Branch weights represent posterior probabilities for clade support. The labelled nodes are the same across figures 2–4 to facilitate comparison.

datasets revealed a monophyletic group of lowland *Adelpha* species (node B, figures 2 and 3) with high clade credibility (BI: 0.98, 1.0; ML bootstrap: 94 and 97%, respectively), but with less resolution among the descendent lineages. The *A. serpa* species group (*A. bredowii*, *A. paraena* and *A. seriphia*) is well supported by both datasets (BI: 1.0, ML: 100, in each) and is the sister lineage to a large number of lowland *Adelpha* species. *Limenitis* and the *A. alala* species group form a monophyletic group (BI: 0.98, 1.0; node C, figures 2 and 3), which is distinct from the lowland *Adelpha* lineage (node B, figures 2 and 3) but also reveals ambiguities in the evolutionary relationships of the descendent lineages. The *A. alala* species group (node D, figures 2 and 3) is a well-supported lineage contained within node C, but its relationship to other members of this clade is unclear. Nearctic and Palaearctic species of *Limenitis* form two separate monophyletic groups (node E is unsupported at the 70% ML and 0.95 BI level; figures 2 and 3), with the exception of *L. populi*.

(b) *Analysis of diversification rates*

Uncertainty in the inference of diversification rate variation associated with the random resolution of polytomies was estimated as confidence intervals, with upper and lower bounds corresponding to the tail probabilities for the 0.025 and 0.975 frequentiles, respectively. Significant diversification rate variation was detected across the *Adelpha* tree by the I_c, M_{II}, M_{Σ} and B_1 statistics over the whole *Adelpha* phylogeny, with probabilities (following Bonferroni correction) in the 95 per cent interval ranging from $p = 0.006–0.05$, 0.004–0.06, 0.033–0.11 and 0.060–0.17, respectively. Application of two likelihood-ratio-based tests to locate shifts in diversification rate indicates that a single shift

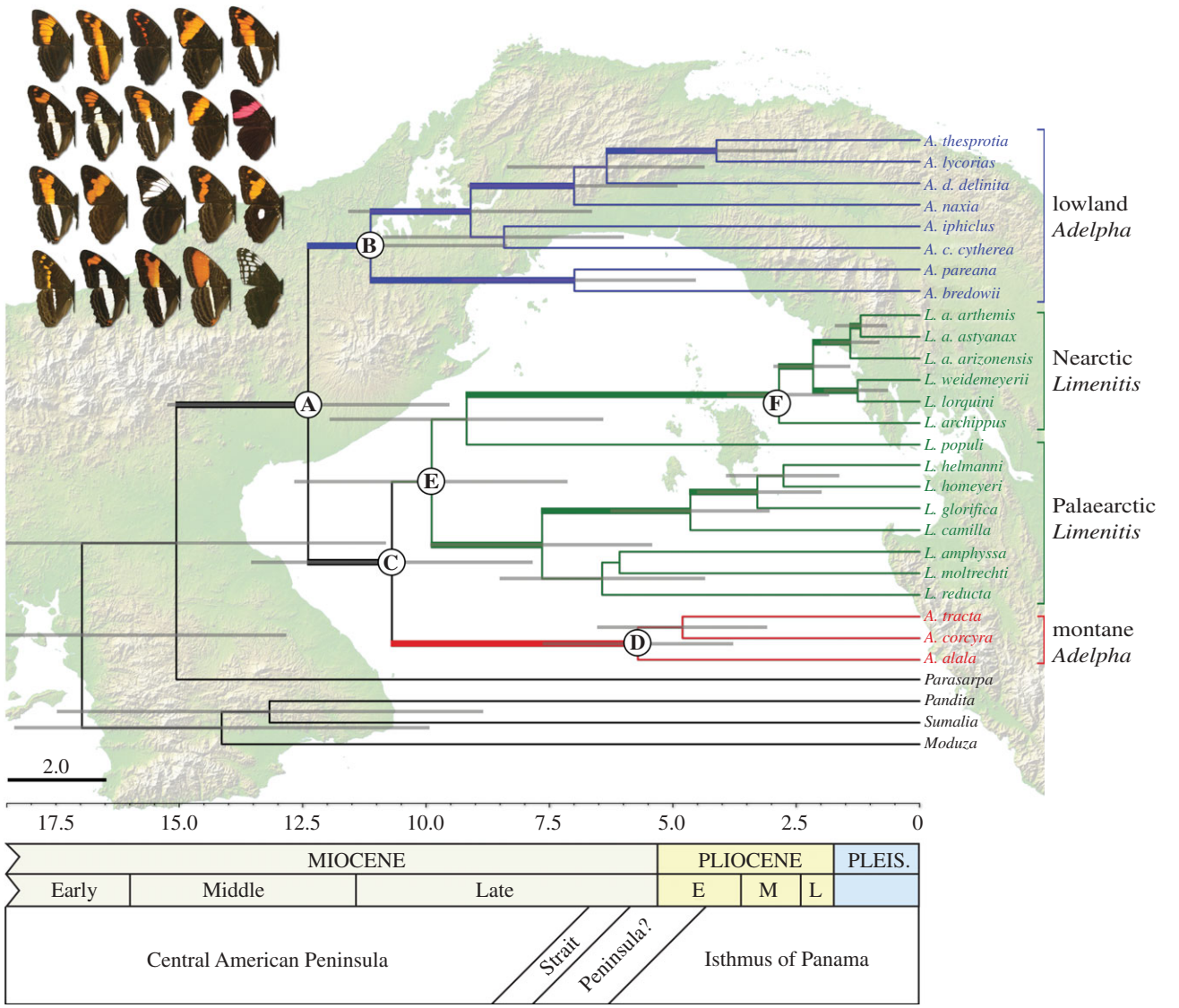

Figure 4. Relative clade divergences resulting from Bayesian inference for *Limenitis* and *Adelpha*. Grey bars represent the 95% confidence intervals associated with estimates of clade ages. Node letters correspond to those in figures 2 and 3. The timeline below the chronogram represents the approximate onset and completion of the Isthmus of Panama. Two major lineages of *Adelpha* (nodes B and C) diversified during the Late Miocene (in which volcanic uplift of the Central American Peninsula preceded collision with South America), and subtend the subgroups of lowland *Adelpha* and the clade containing the *Limenitis* species. This lineage divergence and the consequent radiation of lowland *Adelpha* species also correspond with the detected shift in the diversification rate (figure 2).

in diversification rate occurred along the descending branch from node A (figure 2) to the root branch leading to the lowland *Adelpha* lineage (node B, figure 2; $p\Delta1 = 0.0453$, $p\Delta2 = 0.0537$).

(c) *Timing of divergences*

The Bayesian MCMC estimates of divergence times suggest that *Adelpha* and *Limenitis* shared a common ancestor some 12.5 Myr ago in the Middle Miocene (node A, figure 4). A basal divergence in *Adelpha* gave rise to a clade that radiated throughout the lowland tropics of South America, and secondarily into lower montane habitats, approximately 11 Myr ago (node B), and a second lineage—which is supported by high clade credibility in the BEAST analysis—that remained in temperate or upper montane habitats and retained many of the symplesiomorphic Limenitidini characters (node C). Secondary diversification within this latter lineage may have given rise to the genus *Limenitis* in the Late Miocene, with a subsequent divergence between Nearctic and Palaearctic *Limenitis* taxa occurring soon after (9 Myr ago; node E), with a possible emergence of the Nearctic *Limenitis* sometime within the last 3 Myr (node F).

4. DISCUSSION

The results of the molecular phylogenetic analysis show both similarities and differences with respect to the previous morphology-based hypothesis [22]. Previously well-supported clades were recovered with broadly similar relationships, but (somewhat surprisingly) the small *A. alala* group was recovered as sister to *Limenitis*, rendering *Adelpha* paraphyletic. Four morphological synapomorphies support the *alala* group as sister to all other *Adelpha* [22], so the position of the *alala* group clearly merits further study, with obvious potential implications for taxonomic revision. Most importantly for the

present study, North American and Palaearctic *Limenitis* are robustly supported as the most closely related group to *Adelpha*. Whether *Adelpha* (*sensu stricto*) is monophyletic or not, the data clearly reveal the significant variation in diversification rates across the broader phylogeny, and that the variation arose from a dramatic increase in diversification rate within the lowland *Adelpha* clade.

In this study, we included three of the six montane species in the *A. alala* clade and 35 of the remaining 83 species in the lowland clade. Although both temporal and topological phylogenetic methods are sensitive to incomplete taxon sampling [35–37], our inference of differential diversification is likely to be robust because increased sampling of lowland species of *Adelpha* would presumably increase tree imbalance and estimates of species richness for this clade. Furthermore, we failed to find evidence for major differences in clade age between temperate and tropical American Limenitidini genera. In fact, the Bayesian estimates of divergence time suggest that the common ancestor of both *Adelpha* and *Limenitis* originated about 12.5 Ma, and that both lineages subsequently diversified throughout the Mid-to-Late Miocene (figure 4). Taken together, these results suggest that the observed latitudinal pattern of increased species diversity within *Adelpha* is due to increased diversification in the tropics and is not a consequence of differences in clade age.

Disparity in diversification rates may arise because of differences in extinction rate and/or speciation rate. For example, the marked climatic fluctuations that are regularly experienced by higher latitudes may increase local extinction rates, resulting in a strong impact on latitudinal gradients in species richness [38]. While we do not directly estimate extinction and speciation rates, our results suggest that differential extinction between temperate and tropical lineages does not explain the high diversity observed among lowland species of *Adelpha*. In fact, the shift in diversification rate occurs at the base of the lowland *Adelpha* clade, with the montane *A. alala* group remaining low in diversity despite occupying the same tropical latitudes. Furthermore, if, as our results suggest, the common ancestor of *Adelpha* arrived in South America within the last 15 Myr, then rates of speciation (approx. 90 species) and raciation (approx. 120 subspecies) are among the highest ever documented. An alternative possibility is that increased opportunities for adaptive radiation, especially through diversification in host-plant use, could have resulted in accelerated speciation rates.

The lowland clade of *Adelpha* displays a remarkable breadth in larval host plants, with the family Rubiaceae featuring prominently [21,23,24]. This breadth is in contrast to the temperate and/or montane sister lineage of the *alala* group + *Limenitis*, which feeds mainly on the *Viburnum, Lonicera* and only a few other plant families (e.g. Salicaceae [24]). An early shift to the family Rubiaceae, or another plant family with a diverse tropical lowland flora, was probably important in allowing *Adelpha* species to escape their temperate confines and radiate throughout the neotropical region. Perhaps significantly, the diverse Asian tropical lowland limenitidine genera *Moduza* and *Athyma* also feed on a variety of plant families, including Rubiaceae [24]. In fact, diversity of host use within clades has been demonstrated to be a good predictor of species richness among nymphalid butterflies [39–42], and colonizations of novel host plants are typically associated with historical polyphagy [40]. These findings led Janz *et al.* [43] to propose that diversification of many plant-feeding insects is driven by oscillations in host-plant ranges, which can lead to increased species distributions and, subsequently, opportunities for secondary specialization on novel hosts.

However, although it is tempting to ascribe the increased diversification rates observed within lowland *Adelpha* to an adaptive radiation spurred by new larval host-plant resources, research increasingly suggests that host-plant shifts may be correlated with multiple other ecological changes that independently, or in concert, can also lead to speciation. For example, among mimetic neotropical ithomiine butterflies, host plants are correlated with topography and forest structure [44–46], in addition to butterfly flight height and warning colour pattern [47,48]. *Adelpha* are also apparently involved in mimicry, within the clade as well as with several genera (e.g. *Agrias* and *Doxocopa*) in other nymphalid subfamilies [21,23].

Mimicry is also common among the species of the closely related, temperate genus *Limenitis* [49–53] and, interestingly, frequently results from modification of the same forewing markings that are also often involved in the sharp phenotypic shifts between *Adelpha* subspecies. Mimicry shifts among divergent wing pattern races of *Heliconius* butterflies produce extrinsic post-zygotic isolation owing to selection against hybrid phenotypes [54,55], and it seems likely that mimetic interactions have played at least some role in the rapid phenotypic diversification seen among lowland *Adelpha* species.

Finally, our estimate of divergence times for these butterfly lineages significantly pre-dates the rise of the Isthmus of Panama (approx. 3.5 Ma), raising the question of how the common ancestor of the lowland clade managed to invade South America. Recent lithostratigraphic, biostratigraphic and chemostratigraphic analyses of Panamanian geological formations indicate that a terrestrial connection existed between Panama and North America as early as 19 Ma in the form of a high-relief volcanic peninsula, which persisted throughout much of the Miocene (see electronic supplementary material) [56]. Although the Atrato Seaway continued to separate the Central American peninsula from South America until the final formation of the Isthmus, the two land masses remained in close proximity, separated by as little as 100 km, for the majority of the Miocene. Thus, it is possible that the colonization of South America by the lineage of *Adelpha* that subsequently diversified throughout the lowland Neotropics occurred via short-distance dispersal among volcanic islands, or perhaps over somewhat larger distances of water, sometime during the Middle Miocene. In contrast, it appears that the less speciose, montane lineage of *Adelpha* diversified in the Late Miocene (5–3 Ma; figure 4), and may represent an independent invasion of South America by way of the Isthmus of Panama.

5. CONCLUSIONS

Ultimately, variation in patterns of species richness among temperate and tropical regions is the result of

differential rates of speciation, extinction and the unique biogeographic history of lineages [57]. We have shown that the dramatic diversity of lowland *Adelpha* communities is the result of a rapid diversification following colonization of the neotropical lowlands, rather than a long history of evolution. Furthermore, our results are consistent with the hypothesis that early shifts onto novel host plants may have been a key innovation driving the adaptive radiation among *Adelpha* butterflies. If so, then it is likely that strong natural selection resulting from geographical differences in the pattern and magnitude of species interactions [14] has played an important role in promoting rapid adaptation, coevolution and speciation within this genus.

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