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 cladres 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 alula group, resembles remaining Limenitidini genera in North America, the Palaearctic and temperate Asia in having...
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

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.
conducted with RAxML v. 7.2.6 [26,27] for locus-specific and multilocus datasets. ML inferences were 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 \( (M_0) \) and sum \( (M_2) \) 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.

(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 \((\text{cox1}, \text{ef1}-\alpha, \text{vog}, \text{gapdh}, \text{idh} \text{ and } \text{rps}5)\), 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 branches \((\text{cold and four incrementally heated}) \) 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 Symmetric TREE v. 1.1 [31,32]. Whole-tree diversification rate variation was estimated with two rate-shift statistics \((M_0) \) and \( (M_2) \) 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

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 vog dataset
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...
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 descendant 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 D, 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 descendant 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 Ic, Mf, Ms and B1 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...
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; $pD_1 = 0.0453$, $pD_2 = 0.0537$).

(e) 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 racion (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, Lonicer 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 Medusa 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 polyphyly [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-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 chronostratigraphic 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 (3–3 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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REFERENCES

30. Nylander, J. A. A. 2004 MrModelTest v2. Program distributed by the author, Department of Systematic Zoology, EBC, Uppsala University, Sweden.


