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Rapid diversification associated with ecological specialization in Neotropical *Adelpha* butterflies

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Abstract

Rapid diversification is often associated with morphological or ecological adaptations that allow organisms to radiate into novel niches. Neotropical *Adelpha* butterflies, which comprise over 200 species and subspecies, are characterized by extraordinary breadth in host plant use and wing colour patterns compared to their closest relatives. To examine the relationship between phenotypic and species diversification, we reconstructed the phylogenetic history of *Adelpha* and its temperate sister genus *Limenitis* using genomewide restriction-site-associated DNA (RAD) sequencing. Despite a declining fraction of shared markers with increasing evolutionary distance, the RAD-Seq data consistently generated well-supported trees using a variety of phylogenetic methods. These well-resolved phylogenies allow the identification of an ecologically important relationship with a toxic host plant family, as well as the confirmation of widespread, convergent wing pattern mimicry throughout the genus. Taken together, our results support the hypothesis that evolutionary innovations in both larvae and adults have permitted the colonization of novel host plants and fuelled adaptive diversification within this large butterfly radiation.

Keywords: host shift, mimicry, phylogenetics, RAD-Seq, speciation

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Introduction

Adaptive radiations are episodes of diversification, characterized by rapid speciation and ecological specialization (Schluter 1996, 2000; Losos 2010), that occur when natural selection drives divergence in response to ecological opportunity (Dobzhansky 1948; Simpson 1953; Gavrilets & Losos 2009; Schluter 2000; Losos 2010). While adaptive radiations are often associated with the evolution of key innovations or competitive release (Simpson 1953), identifying the evolutionary mechanisms promoting speciation can be complicated by the challenge of reconstructing the evolutionary history of rapidly diverging lineages (Glor 2010).

Clades of phytophagous insects that display signatures of adaptive radiation often share complex evolutionary histories with their host plants. Among nymphalid butterflies, patterns of species richness are strongly correlated with diversity of host plant use, and historical host shifts have been shown to precede increases in diversification rate in over a dozen lineages (Fordyce 2010; Janz *et al.* 2001; Janz & Nylin 2008). Although colonization of novel hosts may directly lead to reproductive isolation and sympatric speciation within insects (Berlocher & Feder 2002; Drès & Mallet 2002; Janz *et al.* 2006), patterns of sustained diversification within lineages may also be explained by the coevolution of defensive and exploitative traits in host plants and their herbivores (Ehrlich & Raven 1964; Strong *et al.* 1984). For example, plants commonly

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employ secondary metabolites, such as alkaloid compounds, to deter insect predators (Fraenkel 1959; Ehrlich & Raven 1964; Berenbaum 1990). Over time, herbivores that evolve resistance to these compounds may sequester them in their body tissues and subsequently evolve bright warning coloration to signal their unpalatability to visual predators (Holzinger & Wink 1996; Ruxton *et al.* 2004). These warning patterns may be mimicked in turn by unrelated species, spurring further phenotypic diversification and speciation (Willmott & Mallet 2004; Jiggins 2008; Mallet 2009). Consequently, the evolutionary 'arms race' between plants and herbivores may lead to frequent phenotypic shifts and species radiations that shape a substantial fraction of the diversity of modern organisms (Ehrlich & Raven 1964; Berlocher & Feder 2002).

Adelpha butterflies (family Nymphalidae), commonly referred to as 'sisters', range from the northwestern United States to Uruguay, displaying striking latitudinal and elevational gradients in species richness (Willmott 2003a; Fig. 1). Representing one of the largest radiations of Neotropical butterflies, the genus comprises over 200 described species and subspecies, with species richness peaking at the base of the eastern Andes (Willmott 2003a; Fig. 1). Host plant use is remarkably diverse among *Adelpha*, spanning at least 22 plant families with little to no specialization at the host species level (Willmott 2003a). Host plant and morphological diversity, however, is primarily limited to the large lowland clade, one of two major *Adelpha* clades. Previous work has shown that the smaller clade, the montane '*alala* group', is genetically and ecologically distinct from the lowland clade. Like the temperate genus *Limenitis*, the *alala* group comprises a small number of species with relatively limited host plant breadth (Willmott 2003a,b; Mullen *et al.* 2011) and may in fact be less closely related to the lowland *Adelpha* clade than to *Limenitis*, the only other genus in the subtribe Limenitidina in the New World (Mullen *et al.* 2011).

A previous effort to understand the disparity between the hyperdiverse *Adelpha* and less diverse *Limenitis* found evidence that the colonization of the Neotropical lowlands from a temperate ancestor was associated with an increase in diversification rate (Mullen *et al.* 2011). This result is consistent with the hypothesis that early shifts onto novel host plants may have sparked the diversification of *Adelpha*. However, the remarkable similarity of wing colour patterns among many sympatric *Adelpha* species (Fig. 1a) led Aiello (1984) to speculate that the genus as a whole constitutes multiple mimicry complexes, with taxa that specialize on toxic Rubiaceae hosts serving as wing pattern models for mimics both within and outside *Adelpha* (e.g. some *Prepona* and *Doxocopa* species). Mimicry of warning patterns is well documented throughout North American *Limenitis*, where it contributes directly to regional differentiation and subspeciation (Brower 1958; Platt *et al.* 1971; Ritland 1991, 1995; Mullen *et al.* 2008).

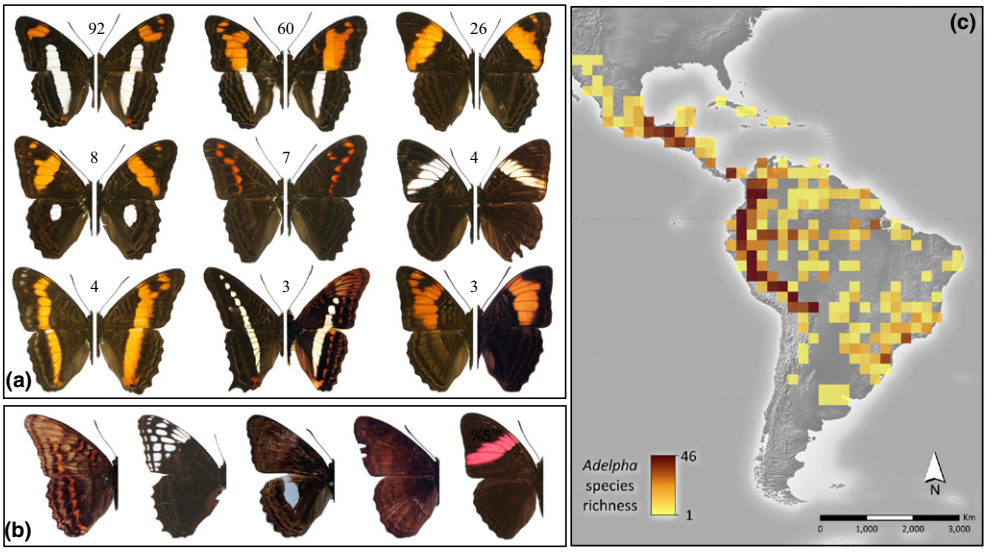

Fig. 1 *Adelpha* wing pattern and species diversity. (a) Examples of the nine *Adelpha* mimicry types. The number above each image indicates the number of species and subspecies with the pattern. From top left: *A. iphiclus iphiclus*, *A. naxia naxia*, *A. thesprotia*, *A. cocala cocala*, *A. salmoneus colada*, *A. boreas boreas*, *A justina justina*, *A. zina zina*, *A. levona*, *A. rothschildi*, *A. epione agilla*, *A. lycorias wallisii*, *A. ethelda ethelda*, *A. leuceria juanna*, *A. gelania gelania*, *A. seriphia barcanti*, *A. mesentina mesentina*, *A. melona deborah*. (b) Five species have a unique wing pattern. From left: *A. seriphia egregia*, *A. demialba demialba*, *A. justina inesae*, *A. zina pyrczi*, *A. lycorias lara*. (c) *Adelpha* species richness across the Neotropical region (modified with permission from Mullen *et al.* 2011).

Therefore, natural selection related to mimetic wing pattern phenotypes and colonization of novel host plants may both have played an important role in the rapid phenotypic diversification of lowland *Adelpha* species. Tests of this hypothesis, however, have been difficult to perform in the absence of a well-resolved phylogeny (see Glor 2010). Previous efforts to resolve the phylogeny of this group based on morphology and the sequences of several genes have generated inconclusive support for many putative clades, as well as unresolved polytomies (Mullen *et al.* 2011; Willmott 2003b).

Here, we use a phylogenomic approach to (i) resolve species-level relationships among *Limenitis* and *Adelpha* butterflies; (ii) reconstruct the history of host plant specialization and wing pattern evolution across this radiation; and (iii) test the hypotheses that historical changes in larval host plant use and the origin of novel wing pattern phenotypes correspond with shifts in diversification rates in *Adelpha*. The combination of genuswide phenotypic data and genomewide markers allow for the identification of key phenotypic shifts, across multiple species and life stages, that have contributed to this rapid butterfly radiation.

Methods

Sampling and molecular methods

Adelpha samples were collected between 2000 and 2012 at 12 sites in the Ecuadorian Andes (300–1650 m) and four sites in Oaxaca, Mexico (380–2000 m) (Table S1, Supporting information). *Limenitis* and Limenitidinae out-group samples were collected between 1999 and 2002 from sites in the United States, Europe, Russia and South-East Asia (Mullen 2006; Table S1, Supporting information). Given the large geographic range of *Adelpha* and the rarity of many endemic species and subspecies (Willmott 2003a), well-preserved tissue samples were obtained for only 43 of the 85 total species. Whenever possible, two individuals from each species, including distinct subspecies, were selected for fragment library construction. Genomic DNA was extracted from butterfly thorax muscle and/or abdominal tissue from a total of 125 butterflies using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Corp., Valencia, CA, USA) (Table S1, Supporting information). For 15 samples with insufficient DNA (<500 ng), we used whole-genome amplification (REPLI-g Mini Kit, Qiagen) prior to RAD library preparation, but only six genome-amplified samples generated sufficient data to be included in the final phylogenetic data set (Table S1, Supporting information).

Double-digest RAD-Seq libraries were prepared following DaCosta and Sorenson (2014; see also Hohenlohe *et al.* 2012). Briefly, 500 ng of genomic DNA was digested with two methylation-insensitive restriction enzymes, *BfuCI* and *PstI* (New England Biolabs Inc., Ipswich, MA, USA), which were chosen to yield *c.* 10^5 loci based on an *in silico* digest of the *Heliconius melpomene* genome. Adapters containing sample-specific barcodes and TruSeq Illumina primers (Illumina Inc., San Diego, CA, USA) were ligated to the resulting sticky ends, and fragments in the 300- to 450 base-pair size range, including adapters, were excised from a low-melt agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen Corp.). Fragments were then PCR-amplified for 23 cycles, using primers that incorporated Illumina indices, with Phusion® High-Fidelity DNA polymerase (New England Biolabs Inc.). Products from each sample were purified using Agencourt AMPure XP beads (Beckman Coulter Inc, Indianapolis, IN, USA), quantified with qPCR (KAPA Biosystems, Wilmington, MA, USA) and finally pooled in equimolar amounts. Single-end, 150-bp sequences were generated on an Illumina HiSeq 2500 two-lane, rapid run flow cell.

Data processing

Demultiplexing, filtering and clustering of sequence reads were performed with the inclusive *pyRAD* software pipeline (Eaton 2014; Eaton & Ree 2013). Restriction sites and adapters were trimmed, and reads were assigned to individuals based on combinatorial indexing (see Peterson *et al.* 2012; Table S5, Supporting information). Bases with Phred quality scores <20 were recorded as missing (Ewing *et al.* 1998; Ewing & Green 1998), and reads with >10 Ns were discarded.

Filtered reads with 85% sequence similarity or greater were then clustered into putative loci within samples using the USEARCH algorithm (Edgar 2010). Sequencing error rate and heterozygosity were estimated and used to create consensus sequences for each cluster. The binomial probability of hetero- or homozygosity at each site was calculated and used to make genotype calls, and nonsignificant sites were assigned Ns. We retained only clusters from which genotypes could be reliably called (fewer than 10 Ns and >8x depth) and which were unlikely to be repetitive elements or paralogs (depth <1 standard deviation from mean for non-amplified samples). Consensus sequences were then clustered across samples at 85% similarity and aligned with MUSCLE v3.8.31 (Edgar 2004). To further filter for paralogs, clusters (i.e. putative loci) in which >15% of individuals shared a heterozygous site were discarded. For loci passing the above filters, one allele per individual was retained for downstream analyses, with random sampling of one of the two haplotypes in heterozygous individuals.

Exploration of missing data

Examination of the data revealed that only a fraction of the total number of loci was recovered for most individuals (see results). To explore the effect of missing data on phylogenetic inference, custom Python scripts were used to assign each locus to one of seven partitions (Table 1) based on the number of taxa without sequence data at that locus. Sequences from all loci within each partition were concatenated and analysed in RAxML v.8.0.19 under the GTRGAMMAI model with 100 fast bootstrap replicates (Stamatakis 2014; Stamatakis *et al.* 2008), and the seven resulting trees were compared using the Tree Farm package in MESQUITE v.2.75 by calculating the proportion of shared clades and the patristic distance correlation (Maddison *et al.* 2011). Based on these results (see below), the 12 528 loci from partitions 1–6 were utilized for additional phylogenetic analysis.

Phylogenetic methods

Initial tree searches were performed using all samples to confirm monophyly of species duplicates. Subsequently, to speed computation, duplicates with more missing data were removed (Table S1, Supporting information), such that each species was represented by the individual with more data. Four samples with high levels of missing data had universally low bootstrap support and inconsistent phylogenetic placement among data partitions, so these samples were also removed, resulting in a final data set of 66 samples representing 61 species of *Adelpha* and *Limenitis* plus several outgroups (Table S1, Supporting information). A final ML analysis was performed as above on 12 528 concatenated loci totalling 1.75 million alignment positions, with an average of 79.5% missing data per individual.

Bayesian analysis was performed in BEAST v. 2.1.1 under a GTR+I+Γ substitution model, a relaxed lognormal clock and a birth–death coalescent prior (Bouckaert *et al.* 2014). The tree was calibrated by specifying that the age of the common ancestor of *Adelpha* and *Limenitis* be drawn from a normally distributed prior ($\mu = 12.5$ mya; $\sigma = 1.8$), based on previous studies of this group that established maximum and minimum time constraints with host plant and butterfly fossil ages (Wahlberg *et al.* 2009; Mullen *et al.* 2011). Simultaneous analysis of all data from the complete supermatrix (12 528 loci and 1.75 million base pairs) was not computationally feasible. Thus, we conducted twelve independent, replicate tree estimates on subsets of 1000 concatenated loci (*c.* 145 000 bp) randomly selected from the total data set, with replacement between subsets. Each run was terminated after a minimum of 5 million generations postconvergence, as assessed using Tracer v1.6.0 (average run length = 9.9 million generations) (Rambaut *et al.* 2013). The last 5 million generations of each run, with trees sampled every 3000 generations, were combined with LogCombiner v2.1.1 (Rambaut & Drummond 2014) to generate a total posterior distribution containing 20 000 trees. Memory constraints required additional subsampling, such that 8042 trees were randomly sampled without replacement from this distribution. TreeAnnotator v2.0.3 (Rambaut & Drummond 2013) was used on the distribution of 8042 trees to calculate the final maximum clade credibility tree (hereafter 'Bayesian consensus tree'), including the posterior probabilities of clades.

To avoid limiting our analysis solely to concatenated sequence data, which ignores potential discordance among the evolutionary histories of individual genes from, for example, incomplete lineage sorting (Gadagkar *et al.* 2005; Edwards *et al.* 2007; Kubatko & Degnan 2007; Rannala & Yang 2008), we employed three alternative approaches to phylogeny estimation. First, a species tree was estimated using a set of 12 528 individual gene trees with NJst on the species tree analysis web (STRAW) server (Liu & Yu 2011; Shaw *et al.* 2013). NJst calculates the average number of internodes between all pairs of species across the unrooted input gene trees; this 'species tree' method is advantageous because it synthesizes data from gene trees with independently estimated parameters, eliminating potential biases resulting from applying a single model to all concatenated loci (Rannala & Yang 2008). Here, randomly ordered gene trees (Guindon *et al.* 2010) were generated with PHYML v3.0 under the HKY substitution model

Table 1 Loci divided into partitions based on the number of taxa missing data

Partition	Taxa without data	Loci	Characters	Missing characters (%)	Mean ML Bootstrap
1	0–19	175	25 287	24.3	93.9
2	20–29	337	48 574	39.6	94.2
3	30–39	746	107 926	55.0	92.3
4	40–49	1880	272 394	69.9	94.8
5	50–56	4142	596 665	81.9	93.9
6	57–60	5248	750 929	89.0	91.8
7	61–63	48225	6 904 713	94.8	66.7

(see J. M. DaCosta & M. D. Sorenson *submitted;* for details). Additionally, 223 169 binary SNPs were scored from the 12 528 loci, and those present in at least 5% of taxa were used to estimate a species tree in RAxML under the ASC_GTRGAMMA model, which conditions the likelihood calculations on all characters being variable (Lewis 2001; Stamatakis 2014). Finally, the 12 528 loci were scored for the presence or absence of data for each individual, and this presence–absence matrix was analysed in RAxML under the BINGAMMAI model.

Character evolution

Host plant usage data were primarily collected from Willmott (2003a), Scott (1986) and HOSTS, an online global database of lepidopteran host plants maintained by the Natural History Museum, London (Robinson *et al.* 2010). Most sampled *Adelpha* species were classified by their wing pattern as belonging to one of nine putatively mimetic groups (Fig. 1a) (Willmott 2003a), with the exception of *A. lycorias lara* (Fig. 1b). Categorical host plant and wing pattern characters were mapped on the likelihood and Bayesian consensus trees with MESQUITE, and ancestral states along the tree were inferred using both parsimony and maximum likelihood (Maddison and Maddison, 2011).

Diversification rate analyses

To begin evaluating the hypothesis that host shifts and/or mimicry has contributed to increased diversification in *Adelpha*, three tests for diversification rate heterogeneity were applied to the Bayesian consensus tree. First, SymmeTREE v1.1 (Chan & Moore 2005) was used to calculate seven statistics comparing observed branching patterns across the tree with those expected under a null, equal-rates Markov model. Second, BayesRate v1.63 (Silvestro *et al.* 2011) and BAMM v2.0 (Rabosky 2014) were each used to compare the marginal likelihoods of models estimating one rate or two rates across the tree (i.e. the likelihood of no rate shifts versus one rate shift). BayesRate performs likelihood calculations using thermodynamic integration, which, unlike traditional harmonic mean estimates, is not biased towards more parameter-rich models (Lartillot & Philippe 2006). BAMM implements an exponential change function to determine the most likely number of rate shifts across a tree. Both of these methods can correct for incomplete sampling when the proportion of sampled taxa is specified, under the assumption that species are missing at random from designated parts of the tree. In contrast, the topological tests in SymmeTREE cannot account for missing taxa; however, nearly all unsampled *Adelpha* species belong to the lowland clade (Willmott 2003b), and their inclusion should only increase estimates of diversification imbalance. Finally, to specifically test whether phenotypic shifts corresponded with increased diversification in *Adelpha*, the null Markov model in SymmeTREE was also used to test each branch of the tree as the location of a shift in diversification rate.

The results from these diversification rate tests, when compared to ancestral character estimations, suggested that a single rate shift coincided with a major host plant shift (see results). Consequently, the tree was divided into two sections (before and after the shift) to explicitly test for different diversification rates in BayesRate under a pure-birth, two-rate model with a uniform diversification prior. A pure-birth model was used because no extinction information is available in this group, and likelihood comparisons in BayesRate implied that a pure-birth model best fits the observed data. Therefore, to independently estimate speciation rates for each section, a search of 50 000 generations was performed on 175 random trees from the Bayesian posterior distribution, sampling every 2500 generations after 30 000 generations of burn-in.

Results

Sequencing

A total of 230.4 million sequence reads were generated, of which 156.9 million passed stringent quality filters. We retained an average of 1.33 million reads per sample (range: 0.56–3.81 million; Table S1, Supporting information). After removing species duplicates, clustering within samples produced an average of 7468 loci per individual (range: 597–15 008; Table S1, Supporting information). There was no relationship between number of reads and number of loci ($R^2 = 0.0074$; $P = 0.49$), suggesting that sampling depth was sufficient to recover most of the ddRAD loci represented in the fragment library (i.e. *BfuCI-PstI* restriction fragments between 178 and 328 bp in length). However, samples that underwent genome amplification had, on average, *c.* 4500 fewer loci than samples that did not ($t_{123} = 5.37$; $P < 0.001$), and there was a significant negative relationship between the number of recovered loci and the time since collection of a sample ($R^2 = 0.0558$; $P = 0.008$). This suggests that some intended loci were not represented in fragment libraries prepared from lower-quality DNA extracts, particularly in older samples that may have been subjected to additional freeze–thaw cycles. Additionally, recovery of loci and the GC content of recovered loci were negatively correlated ($R^2 = 0.2698$; $P < 0.001$; Fig. S1, Supporting information). This may be due to a proportionally greater effect of GC amplification bias (see DaCosta & Sorenson 2014) in libraries that recovered fewer loci.

In general, sequenced loci were highly variable. On average, each 145-bp locus (±standard deviation, 6.49 bp) contained 28.8 SNPs (±13.00) and 5.09 short indels (±5.50) of length 3.48 bp (±3.13) across the phylogenetic breadth of sampled taxa. For phylogenetic analysis, indels were treated as missing data. Average heterozygosity within individuals was significantly higher in *Adelpha* than in *Limenitis* (0.0085 vs. 0.0060; $P < 0.0001$). There was a strong positive correlation between mean per-locus nucleotide diversity (π) and the number of taxa for which data were missing within data partitions ($R^2 = 0.999$; $P < 0.0001$ for partitions 1–6; Fig. S2, Supporting information). For example, the mean per-locus nucleotide diversity in partition 6 (86–91% of taxa missing data) was almost twice as high as the mean nucleotide diversity of loci in partition 1 (0–29% of taxa missing data), as expected if the gain and loss of restriction sites are correlated with nucleotide diversity within a given genomic region.

Phylogenetics

The RAD data set provided unprecedented resolution of species relationships within *Adelpha* and *Limenitis* (Fig. 2). Nearly all nodes in the ML tree had bootstrap values of 95 or greater. The few nodes with relatively low support all followed short internodes, which may be due to incomplete lineage sorting and/or a limited number of synapomorphies across divergences over short timescales (Fig. 2; Wiens 2008; Rannala & Yang 2008). Support was similarly high in trees derived from a selection of biallelic SNPs, locus presence/absence data and the NJst method (Table 2; Fig. S3, Supporting information).

Our results indicate that the Eurasian and North American *Limenitis* are entirely embedded within the New World *Adelpha*, rendering *Adelpha* paraphyletic. The montane *A. alala* group is sister to *Limenitis*, while the rest of the genus, comprising a large radiation of lowland species, is sister to the *Limenitis/alala* clade (Fig. 2, Node C). While an earlier cladistic analysis identified several morphological synapomorphies for *Adelpha* (Willmott 2003b), the molecular results are conflicting, and description of a new genus for the montane *Adelpha* clade will likely be necessary. However, previous hypotheses of lower-level *Adelpha* relationships based on morphological similarity are largely consistent with our phylogenetic results, particularly within the *alala, serpa* and *phylaca* groups (Willmott 2003b). The improved resolution offered by genomic data, however, indicates that the *iphiclus* and *cocala* groups are not monophyletic and that the few morphological characters previously used to define the polyphyletic *cocala* group are misleading.

Variation in the extent of missing data among partitions had a minimal effect on tree topology and nodal support values. Although the number of recovered loci increased as the minimum number of individuals requiring data to define a locus was decreased (Table 1), partitions 1–6 produced maximum-likelihood (ML) trees that were largely in agreement and well supported (Table 1; Fig. S4; Table S2, Supporting information). Only partition 7, in which nearly 95% of the overall data matrix was missing, recovered a relatively inconsistent and poorly supported topology.

Similarly, trees constructed using different phylogenetic methods were well supported and in strong agreement (Fig. S3; Table S3, Supporting information). One exception involved the placement of *L. sydyi*, which grouped with most other Eurasian *Limenitis* in ML trees but with *L. populi* and the North American *Limenitis* in the NJst and BEAST trees. The evolutionary relationships among five more recently evolved species of Eurasian *Limenitis* (Node I), which are separated by poorly supported nodes and some short branches, were also inconsistent among methods. Additionally, in a few cases, groups of taxa appeared monophyletic in one of the five trees, but branched sequentially off the backbone in the others (e.g. Node F, Fig. 2–3; Fig. S3, Supporting information). In general, however, clade composition and branching order was consistent among trees produced using different phylogenetic methods (Table S3, Supporting information).

The tree constructed from a presence–absence matrix was the least topologically similar to trees produced by other methods, as measured by the overall proportion of shared clades and the correlation of patristic distances across trees (Table S3, Supporting information). This was largely due to the presumably erroneous placement of one *Limenitis* sample and three *Adelpha* taxa as a clade within the out-group taxa (Fig. S3b, Supporting information). These four misplaced samples, collected over a decade before library construction and subjected to whole-genome amplification, had the fewest sequenced loci of all in-group samples (Table S1, Supporting information). Missing data in these individuals were probably due to DNA degradation over time, resulting in a reduction of intact DNA fragments spanning pairs of adjacent restriction sites. The presence or absence of loci among other taxa, in contrast, closely paralleled the relationships inferred from concatenated sequence data (Fig. S3, Supporting information; Fig. 2–3). This suggests that the gain and loss of restriction sites over evolutionary timescales can generate phylogenetically meaningful presence–absence data in a RAD-Seq experiment, as has previously been shown with null alleles from microsatellite markers (Amos 2006), as long as the samples used to generate data are of sufficient quality.

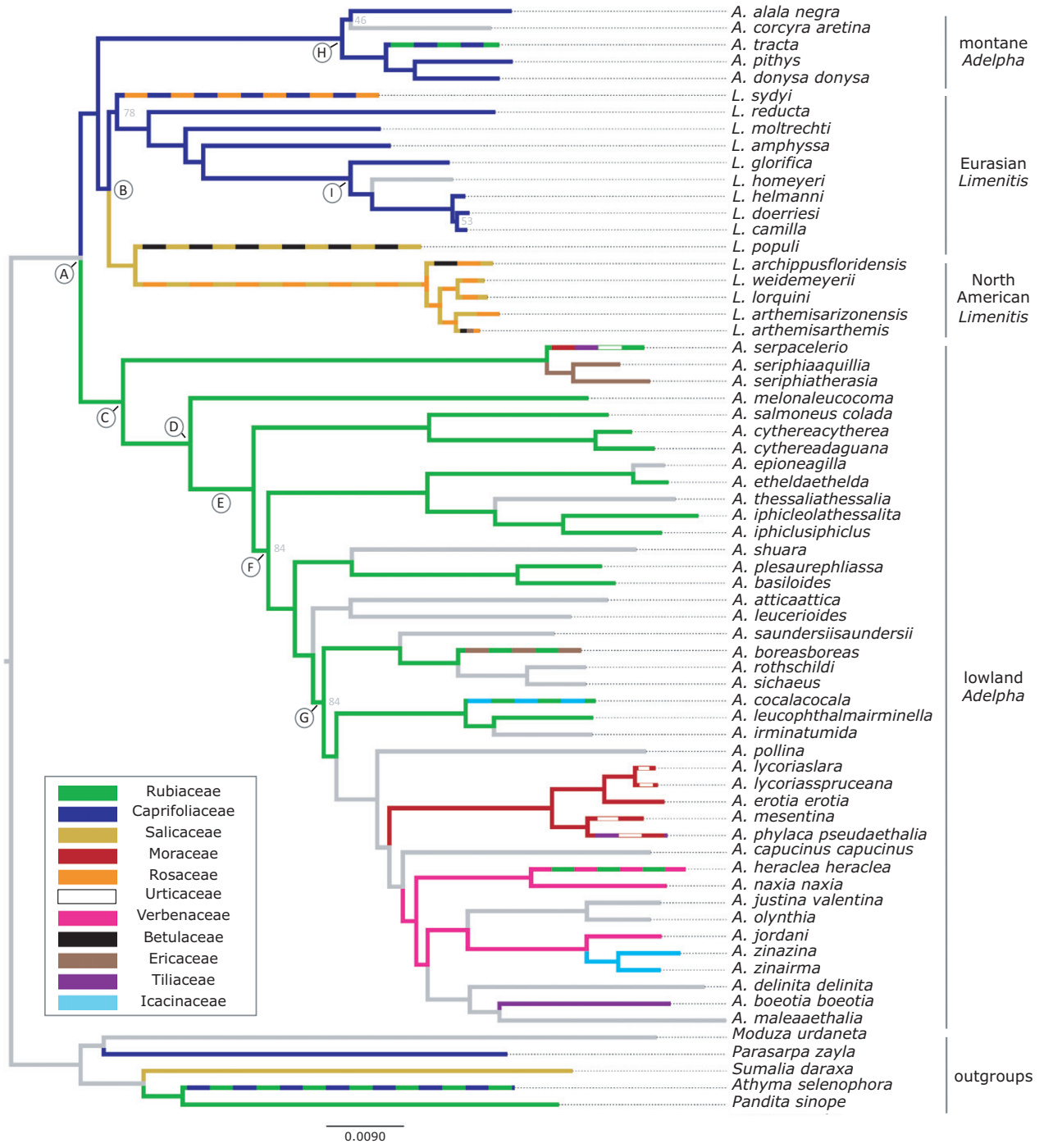

Fig. 2 Maximum-likelihood tree based on concatenated sequence data for 12 528 loci. Nodes with bootstrap values ≥95 are unlabelled. Branches are coloured by host plant family. Grey branches indicate unknown host plant. For clarity, families that host fewer than two taxa are omitted (Table S4, Supporting information). Species groupings are after Willmott (2003a).

Character evolution

Host plant use appears to be quite labile over time, changing states 23 times in the history of the in-group (Fig. 2; Table S4, Supporting information). The ancestral feeding state for *Limenitis* and the montane *alala* group was found to be Caprifoliaceae, although the North American *Limenitis* shifted to Salicaceae and other families (Fig. 2). The ancestral host family for the lowland

Table 2 Data type and average node support for phylogenetic models applied to RAD-Seq data

Phylogenetic model	Data	Support
GTRGAMMA (RAxML)	12 528 concatenated loci	97.46
GTR+I, lognormal clock, birth-death (BEAST)	12 samples of 1000 concatenated loci	90.06
NJst (STRAW)	12 528 ML gene trees	94.33
ASCGTRGAMMA (RAxML)	223 169 binary SNPs	98.78
BINGAMMA (RAxML)	Presence/absence of 12 528 loci	90.62

Adelpha was Rubiaceae (Fig. 2). Although some outgroups also feed on Rubiaceae, likelihood analysis assigned only 0.46% of the proportional likelihood for host state to Rubiaceae on the branch leading to *Limenitis* and the montane *Adelpha*. However, Rubiaceae constituted 64.9% of the proportional likelihood on the branch leading to the entire lowland *Adelpha* clade, increasing to 98.8% for the branch between nodes C and D and 99.996% for branch E (Fig. 2). Finally, although host plant data for *Adelpha* species are incomplete, some derived *Adelpha* species appear to have specialized on other families, including Moraceae and Verbenaceae (Fig. 2).

Adelpha dorsal wing patterns have also been highly labile, changing states 24 times among the 46 taxa in the tree (Fig. 3). The most common pattern in the genus, shown in red, is ancestral for the *alala* and *serpa* groups. In contrast, a modified pattern, shown in blue, is inferred as the ancestral state across most of the large lowland *Adelpha* clade. Nonetheless, *alala*-like wing patterns reappear six times in the large clade, despite nearly 12 million years of divergence between the two clades (Fig. 3, Table 3, Node A). In fact, all patterns for which we sampled more than one representative species, including those indicated by green and purple in Fig. 3, appear to have had multiple independent origins in the lowland *Adelpha*.

Diversification rates

Our Bayesian age estimates of nodes (Table 3) have substantially narrower 95% credible ranges than earlier estimates of divergence times, which used a more limited data set and wider crown age priors (Pohl *et al.* 2009). Our age estimates thus allow for a relatively more precise calculation of the net diversification interval (NDI) within clades. The NDI represents the average amount of time between the origin of a lineage and its subsequent branching into two lineages (Coyne & Orr 2004), assuming the increase in net species number is exponential. The estimated NDI is 2.73 million years for the lowland *Adelpha* clade (95% HPD, 1.83–3.49 my), 3.73 million years for *Limenitis* (2.39–4.66 my) and 6.34 million years for the montane *Adelpha* (4.06–7.92 my).

Consistent with variation in NDI, all seven test statistics implemented in SymmeTREE rejected the hypothesis of an equal-rates Markov random branching model for the Bayesian consensus tree (Fig. 3; $P = 0.0004$–0.01), representing strong statistical evidence for diversification rate heterogeneity across the tree. Relatively short branches between species groups in the large lowland *Adelpha* clade (e.g. Fig. 2, Branch G) further support this hypothesis. Analysis by thermodynamic integration in BayesRate also strongly preferred a two-rate model for the tree over a single-rate model (2*difference in log marginal likelihoods = 20.70; Kass & Raftery 1995).

In contrast, the BAMM analysis could not distinguish between the likelihoods of models with various numbers of rate shifts (e.g. for 0 versus 1 shifts, $2*ln(mL) = 1.98$). However, BAMM is a macroevolutionary method that has commonly been applied to deeper or more broadly sampled trees (e.g. root age of tens or hundreds of millions of years; family-level sampling; Rabosky 2014; Rabosky *et al.* 2014; Huang & Rabosky 2014; Cook *et al.* 2014; Weber & Agrawal 2014; Schwery *et al.* 2014). Thus, it is possible that our relatively shallow phylogenetic sample may not provide sufficient power for this method.

A single branch near the base of the lowland *Adelpha* (Fig. 3, Branch E), following the divergence of *A. melona leucocoma*, was supported as the location of a diversification rate shift by two branch-specific test statistics in SymmeTREE (Δ_1: $P = 0.033$; Δ_2: $P = 0.042$). Subsequently, we used Branch E (Fig. 3) to divide the tree into two sections to evaluate independent diversification rates. Under the two-rate, pure-birth model implemented in BayesRate, the mean estimated speciation rate was significantly higher for 'postshift' taxa than for 'preshift' taxa (0.307 vs. 0.250 species/million years; $t(1835) = 18.53$; $P < 0.0001$).

Discussion

Phylogenetics

Sequenced RAD tags from *Adelpha* and *Limenitis* formed the basis for well-supported and robust phylogenetic inference from a variety of methods (Table 2). Despite the potential pitfalls of concatenation identified in previous studies (e.g. Kubatko & Degnan 2007; Wagner *et al.* 2013), we found that trees constructed from

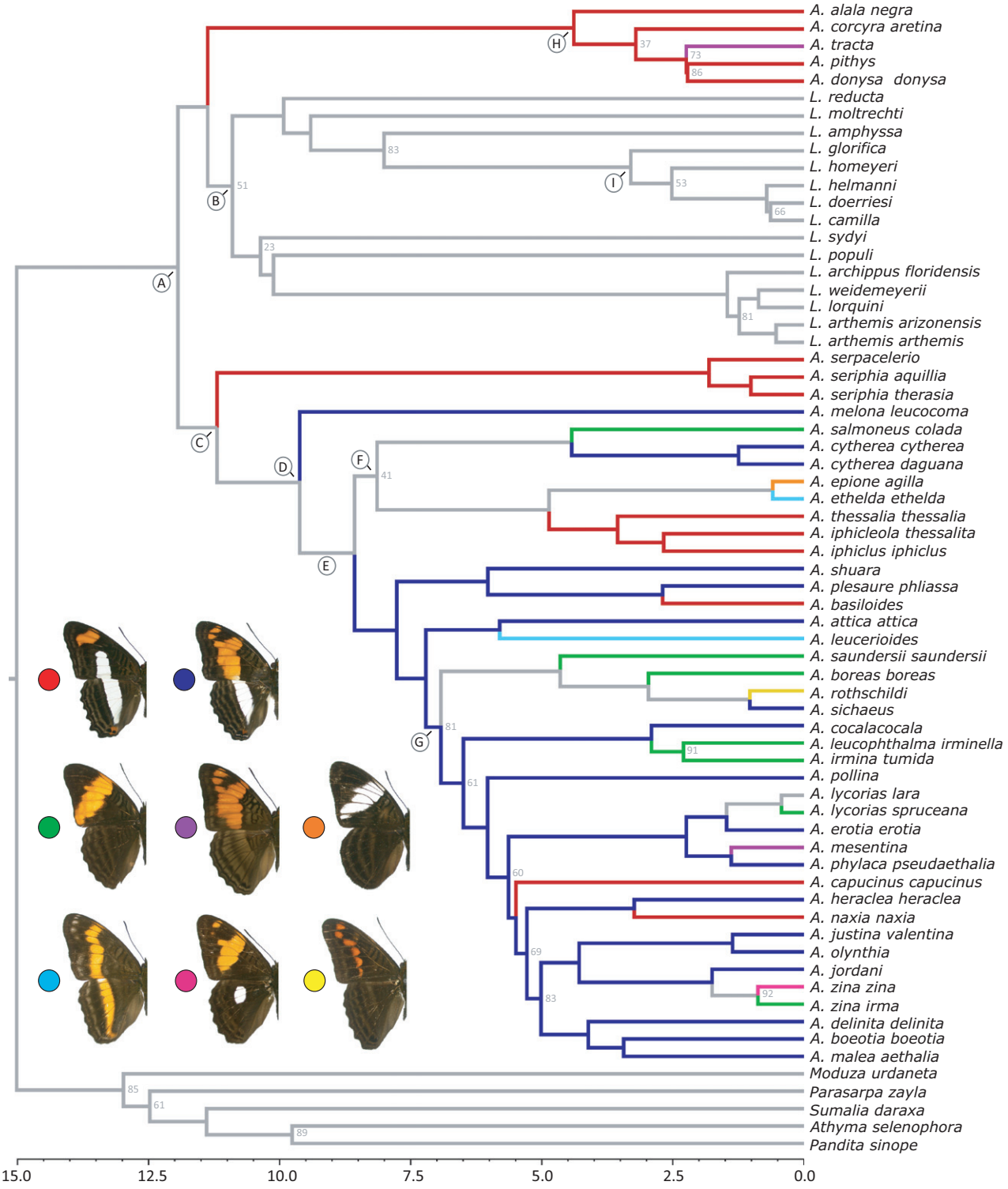

Fig. 3 Bayesian maximum clade credibility tree based on 12 independent samples of 1000 loci from the total of 12 528 loci. Nodes with posterior probabilities ≥95 are unlabelled. Branches are coloured by mimetic *Adelpha* wing pattern type.

concatenated data, SNPs and gene trees produced largely congruent results, in agreement with other recent RAD-Seq studies (Cariou *et al.* 2013; J. M. DaCosta & M. D. Sorenson *submitted*). Although the topology of the NJst tree differed from other analyses for recent divergences (Fig. S3b, Table S3, Supporting information),

Table 3 Bayesian age estimates for labeled nodes (Fig. 3)

Node	Estimated age (my)	95% HPD
A	11.94	8.00–15.26
B	10.90	7.06–13.77
C	11.19	7.54–14.42
D	9.62	6.50–12.68
F	8.14	5.59–10.79
H	4.39	2.39–6.66
I	3.30	1.45–4.43

these shallow differences are unlikely to influence diversification rate tests. Similarly, with the exception of a few low-quality extracts, our phylogenetic results were consistent across a tree constructed from presence–absence data alone and from data partitions spanning a broad range of missing data (Fig. S4, Fig. S3b, Supporting information).

RAD-Seq has been increasingly employed to generate phylogenies from empirical data, including in *Heliconius* butterflies (Nadeau *et al.* 2012), fish (Wagner *et al.* 2013; Jones *et al.* 2013), primates (Bergey *et al.* 2013), ground beetles (Cruaud *et al.* 2014; Takahashi *et al.* 2014) and oaks (Hipp *et al.* 2014). Other *in silico* phylogenetic studies have also demonstrated the utility of RAD-Seq for resolving divergences, as long as they are not too old (Cariou *et al.* 2013; Rubin *et al.* 2012). However, the minimum amount of RAD-Seq data necessary for phylogenetic inference remains an open question, with most studies simply eliminating loci with missing data beyond an arbitrary threshold (Nadeau *et al.* 2012; Jones *et al.* 2013; Cruaud *et al.* 2014; Hipp *et al.* 2014).

Mutations that change the relative locations of restriction sites have been shown to produce null alleles that bias estimates of diversity in population genetic studies (Chapuis & Estoup 2007; Arnold *et al.* 2013; Gautier *et al.* 2013). In contrast, for phylogenetic questions, two studies have demonstrated that larger matrices with higher amounts of missing data produce the most robust trees (Rubin *et al.* 2012; Wagner *et al.* 2013). However, in one study that rigorously investigated the differences among phylogenies constructed from data matrices with variable thresholds for both missing data and sequence similarity within clusters, different thresholds produced markedly different topologies that were individually well supported (Takahashi *et al.* 2014). Although allowing more missing data yielded more highly supported trees within a particular radiation, the independent roles of clustering similarity and missing data thresholds were not specifically determined (Takahashi *et al.* 2014).

Here, we find that missing data among 12 528 loci do not strongly impact the phylogenetic result. In particular, we find that as few as 175 well-sampled, concatenated loci produce a tree largely consistent with trees based on larger data sets (Fig. S4, Supporting information), similar to the results of matrix-size comparisons in Takahashi *et al.* (2014). These results imply that while some threshold for a minimum number of taxa represented by a given locus is required, it may be overly conservative to retain only loci that appear in all or most individuals. Because ddRAD-Seq allows the number of targeted loci to be adjusted by altering restriction enzymes and/or the selected fragment size range, it may be possible to distribute sequence reads over a smaller number of loci in a larger number of species to produce a well-supported and more fully sampled tree.

Diversification rates and host shifts

The results of several diversification rate analyses support rate heterogeneity throughout the tree, consistent with previous findings of a rate shift resulting in an increased rate of diversification in the lowland *Adelpha* clade (Mullen *et al.* 2011). Branch-specific tests indicate that the lineage descended from Branch E (Fig. 2), from which most of the lowland *Adelpha* evolved, has diversified more quickly than other lineages in the tree. The NDI for the lowland *Adelpha* suggests that this clade has diversified at a rate nearly 2.5 times faster than the average estimated rate for arthropods (Coyne & Orr 2004).

Reconstruction of the history of character evolution on the phylogeny indicates that host plant use is diverse across *Adelpha* and *Limenitis*, but that most host shifts occurred early in the evolution of each genus (Fig. 2). We found evidence that the majority of the lowland *Adelpha* use Rubiaceae species as hosts (Fig. 2, Node D) and that Rubiaceae was the ancestral state early in the history of this lineage. This is of particular interest given the well-known production of antiherbivorous, bioactive compounds by members of this plant family (e.g. Phillipson *et al.* 1982; Soto-Sobenis *et al.* 2001; Lopes *et al.* 2004; Aniszewski 2007). Prior to the existence of a robust phylogenetic hypothesis for *Adelpha*, Aiello (1984) tentatively proposed that species whose larvae feed on Rubiaceae might serve as the unpalatable models for other *Adelpha* species. Subsequent work has shown that over three-quarters of Rubiaceae in Panama produce alkaloid compounds known to repel herbivores (Soto-Sobenis *et al.* 2001; Kessler & Baldwin 2002; Schmeller & Wink 1998). Consistent with the hypothesis of chemical defence, each Rubiaceae-specialist *Adelpha* sampled in our study feeds on a genus known to harbour chemicals with poisonous, narcotic or medicinal effects (Schultes 1985; Soto-Sobenis *et al.* 2001; Quattrocchi 2012). Therefore, it seems likely that a

host plant shift to Rubiaceae required physiological adaptation in *Adelpha* larvae to cope with antiherbivorous toxins.

Alternatively, given that Rubiaceae-feeding exists in distantly related out-groups, *Limenitis* and the montane *Adelpha* may have shifted to alternative plant families, rather than the lowland *Adelpha* shifting to Rubiaceae. While additional out-group sampling would be required to distinguish between these two possibilities, the observed association between ancestral host type and diversification rate (Fig. 2) implies that the ability to feed on toxic and diverse hosts has played an important role in the rapid diversification of *Adelpha*.

Mimicry in Adelpha

Our results also suggest that selection for mimicry has shaped wing pattern evolution in both *Limenitis* and *Adelpha*. While mimicry is well known among North American *Limenitis* species with unpalatable models (Brower 1958; Platt *et al.* 1971; Ritland 1991; Ritland & Brower 2000), little is known about the palatability of most *Adelpha* species (but see Srygley & Chai 1990; Pinheiro 1996; Prudic *et al.* 2002). However, strong evidence for mimicry in *Adelpha* includes congruent geographic shifts in wing pattern among comimics, regardless of relationship, and the derivation of similar putative mimetic wing patterns from apparently distinct wing pattern elements (Willmott 2003a). While direct palatability tests of more species are plainly needed, in the light of our well-resolved phylogeny, it is clear that highly similar wing patterns have multiple origins in lineages that are not closely related, both within the diverse lowland clade and across both paraphyletic *Adelpha* clades (Fig. 3). Although we cannot distinguish between repeated *de novo* adaptation and introgression across lineages, as has recently been shown for genes conferring wing pattern mimicry in *Heliconius* butterflies (Dasmahapatra *et al.* 2012; Smith & Kronforst 2013), these results strongly imply that wing patterns in adult *Adelpha* are adaptive and mimetic (e.g. Müller 1879; Mallet & Gilbert 1995; Ruxton *et al.* 2004; Mullen 2006).

Conclusion

Examples of adaptive radiation, which are characterized by rapidly diversifying lineages that become phenotypically and ecologically distinct, present some of the best opportunities to understand how microevolutionary processes acting within populations give rise to diversity across macroevolutionary timescales. Identifying the ecological and evolutionary mechanisms promoting rapid diversification, however, requires reliable and detailed knowledge of the evolutionary relationships among species, which can be muddled by insufficient phylogenetic signal, homoplasy, incomplete lineage sorting or shared ancestral polymorphism at small numbers of loci. Our results demonstrate the utility of genomewide RAD markers for resolving species-level relationships among Neotropical *Adelpha* that were previously confounded by morphological similarity and highly variable wing patterns, here shown to be the result of convergent evolution consistent with widespread mimicry within the genus. We also find phylogenetic evidence for multiple host plant shifts in a rapidly radiating group that, together with the phenotypic evidence for convergence, suggests that natural selection acting at both the larval and adult life stages has contributed to the extraordinarily rapid adaptive diversification of *Adelpha* butterflies.

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E.R.E. and S.P.M. wrote the article. All authors contributed to the design of the study. E.R.E. implemented the methods, and E.R.E., J.D. and M.S. analysed the results. S.P.M., E.R.E., R.I.H., A.D.B. and K.R.W. contributed valuable samples. All authors read and approved this version of the article.

Data accessibility

Raw Illumina reads, phylogenetic tree files, RAD haplotype data for each locus and Python scripts for parsing the data are available from the Dryad Digital Repository. doi:10.5061/dryad.tf704.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 RAD libraries recovering fewer loci have higher GC content.

Fig. S2 Nucleotide diversity plotted by partition (Table 1) reveals a strong correlation between diversity and missing data. Boxed lines indicate median values, with whiskers extending 1.5 times the median in each direction. The width of each box is proportional to the square root of the number of sampled loci.

Fig. S3 Phylogenetic trees for model comparison. (a) ML analysis of 223 169 binary SNPs, (b) ML analysis of presence/absence of 12 528 loci, (c) species tree analysis of 12 528 ML gene trees (Table 2). In (b), the misplaced taxa are indicated with '*'.

Fig. S4 Maximum likelihood trees based on concatenated sequence for loci in each partition (Table 1). Bootstrap value is shown for all nodes.

Table S1 Sample and sequence information for all *Adelpha*, *Limenitis*, and outgroup specimens. Genomic DNA from samples in italics was amplified with the RepliUG Mini Kit. Samples marked in the 'Phylogeny' column were used in the final phylogenetic analysis.

Table S2 Comparisons of ML phylogenetic trees based on unique data partitions (Table 1). Each cell contains the proportion of shared clades and the patristic distance correlation between trees, separated by '/'.

Table S3 Comparisons of phylogenetic trees based on different models and data types (Table 2). Each cell contains the proportion of shared clades and the patristic distance correlation between trees, separated by '/'.

Table S4 Complete host information, at the level of plant family, for sampled *Adelpha*, *Limenitis*, and outgroup species.

Table S5 Identity of barcodes and indices in the adapters added to each sample in the ligation step. Samples can be uniquely identified by a combination of one of each of 32 barcodes and 4 indices.