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Ecological specialization drives rapid diversification in neotropical Adelpha butterflies: a phylogenomic approach

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ECOLOGICAL SPECIALIZATION DRIVES RAPID
DIVERSIFICATION IN NEOTROPICAL *ADELPHA* BUTTERFLIES:
A PHYLOGENOMIC APPROACH

by

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ECOLOGICAL SPECIALIZATION DRIVES RAPID
DIVERSIFICATION IN NEOTROPICAL _ADELPHA_ BUTTERFLIES:
A PHYLOGENOMIC APPROACH

EMILY R. EBEL

ABSTRACT

Adaptive radiations provide exceptional opportunities to examine the relationships between natural selection, adaptation, and speciation. Neotropical _Adelpha_ butterflies may represent such a radiation, characterized by extraordinary breadth in host plant use and wing color patterns. In this study, we use genome-wide RAD markers to reconstruct the complex evolutionary history of _Adelpha_ and the closely related temperate genus, _Limenitis_. Despite the presence of significant missing data, a variety of phylogenetic methods produce similar and highly supported trees. These well-resolved phylogenies allow for the identification of an ecologically important shift to a toxic host plant family, as well as the confirmation of rampant wing pattern mimicry throughout the genus. Taken together, our results support the hypothesis that the colonization of novel host plants represents a key evolutionary innovation that is fueling ongoing adaptive diversification within this large, phenotypically diverse butterfly radiation.
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INTRODUCTION

Adaptive radiations are a form of evolutionary diversification characterized by rapid speciation and ecological specialization (Schluter 1996, 2000; Losos 2010). This process occurs when natural selection drives divergence in response to ecological opportunity (Dobzhansky 1948; Simpson 1953; Gavrilets & Losos 2009; Schluter, 2000; Losos 2010), resulting in increased ecological disparity among closely related species. Although adaptive radiations are frequently associated with the evolution of key innovations or competitive release (Simpson 1953), identifying the evolutionary mechanisms promoting speciation is often complicated by the challenges associated with reconstructing the evolutionary history of rapidly diverging lineages (Glor 2010).

*Adelpha* butterflies, 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 contains over 200 described species and subspecies, with species richness peaking at the base of the eastern Andes (Willmott, 2003a; Fig. 1). Among *Adelpha* species, host-plant use is remarkably diverse, spanning at least 22 families of plants (Willmott, 2003a).

Previous work has shown that one small clade of *Adelpha*, the montane *alala* group, is ecologically and genetically distinct from most of the genus, including in its more limited host plant breadth (Willmott, 2003a, b; Mullen et al., 2011). The *alala* group
Figure 1. *Adelpha* wing pattern and species diversity. (a) The nine mimicry types and their frequencies among *Adelpha* species and subspecies. From top left: *A. iphiclus iphiclus*, *A. naxia naxia*, *A. thesprotia*, *A. cocala cocala*, *A. justina justina*, *A. zina zina*, *A. ethelda ethelda*, *A. leuceria juanna*, *A. mesentina mesentina*, *A. melona deborah*, *A. salmoneus colada*, *A. boreas boreas*, *A. levona*, *A. rothschildi*, *A. gelania gelania*, *A. seriphia barcanti*, *A. epione agilla*, *A. lycorias wallisii*. (b) Five wing patterns are unique to a single species. 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).

more closely resembles the temperate sister genus *Limenitis* in containing only a few species, restricted primarily to the family Caprifoliaceae.

Colonization of novel host plants has long been hypothesized to drive bursts of diversification in phytophagous insects (Erlich and Raven, 1964; Strong et al., 1984; Drès and Mallet, 2002; Janz et al., 2006), and diversity of host use correlates strongly with patterns of species richness among nymphalid butterflies (Fordyce, 2010; Janz et al., 2001; Janz and Nylin, 2008). Previous efforts to understand
the disparity between the hyper-diversity of *Adelpha* and the less diverse *Limenitis* found evidence that the colonization of the Neotropical lowlands by *Adelpha* was associated with an increased rate of diversification (Mullen et al., 2011). In combination with the extraordinary host-plant breadth observed among lowland *Adelpha* species, this result is consistent with the hypothesis that early shifts onto novel host plants may have been a key innovation driving the diversification of *Adelpha*.

However, patterns of host plant utilization are often correlated with other ecological variables, including warning color patterns, which may lead to speciation independently or in concert (Willmott and Mallet, 2004; Jiggins, 2008; Mallet, 2009). Mimicry of warning patterns is well documented throughout North American *Limenitis*, and may contribute to regional differentiation and subspeciation (Brower, 1958; Ritland, 1995; Mullen et al., 2008). The remarkable similarity of wing color patterns among many sympatric species of *Adelpha* (Fig. 1) led Aiello (1984) to speculate that the genus as a whole comprises a large mimicry complex, involving interactions both among *Adelpha* species as well as across genera in other nymphalid subfamilies (e.g. *Agrias* and *Doxocopa*). Interestingly, mimetic wing pattern shifts in *Limenitis* frequently result from modification of the same forewing markings that are often involved in the sharp phenotypic shifts between *Adelpha* subspecies (Willmott, 2003a). Therefore, it is possible that natural selection related to mimetic wing pattern phenotypes has played at least some role in the rapid phenotypic diversification of lowland *Adelpha* species. However, tests of this
hypothesis have been difficult to perform in the absence of a well-resolved phylogeny (see Glor, 2010).

Here, we use a genome-wide RADseq-based phylogenomic approach to 1) confidently resolve the species-level relationships among rapidly diversifying clades of *Adelpha* and *Limenitis* butterflies, 2) reconstruct the history of host plant specialization and wing pattern evolution across this radiation, and 3) to test the hypothesis that shifts in diversification rates correspond with historical changes in larval hostplant use and/or the origin of novel wing pattern phenotypes. Our approach also illustrates the power of RADseq to resolve previously intractable phylogenies, despite the presence of significant missing data, across a variety of modern inference methods.
MATERIALS AND 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 1). Limenitis and Limenitidiine outgroup samples were collected between 1999 and 2002 from sites in the United States, Europe, Russia, and southeast Asia (Mullen, 2006; Table 1). Given the bicontinental range of Adelpha and the rarity of many endemic species (Willmott, 2003a), well-preserved tissue samples could be obtained for only 43 of the 85 total species. However, two individuals from each species, including distinct subspecies, were selected whenever possible for library construction. Genomic DNA was extracted from butterfly thorax muscle and/or abdominal tissue using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Corp., Valencia, CA, USA). Whole-genome DNA from some low-yield samples was amplified with the REPLI-g Mini Kit (Qiagen), though non-amplified samples were preferred in the final phylogenetic data set (Table 1).

Double digest RAD-seq libraries were then prepared from 500 ng of whole-genomic DNA following DaCosta and Sorenson (in review; see also Baird et al., 2008). Briefly, the DNA was digested with two restriction enzymes, BfuCl and PstI (New England Biolabs Inc., Ipswich, MA, USA). Adapters containing sample-specific barcodes and Illumina primers (Illumina Inc., San Diego, CA, USA) were ligated to the sticky ends, and fragments 300-450 base pairs in size, including adapters, were
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selected using gel extraction (Qiagen Corp.). Fragments were then PCR amplified for 23 cycles using Phusion® High-Fidelity DNA polymerase (New England Biolabs Inc.) and purified using Agencourt AMPure XP beads (Beckman Couter Inc, Indianapolis, IN, USA). After quantification with qPCR (KAPA Biosystems, Wilmington, MA, USA) and the Agilent Bioanalyzer (Agilent Technologies Inc., Englewood, CO, USA), individual libraries were pooled in equimolar amounts and sequenced using two single-end 150 bp lanes of Illumina HiSeq 2500.

### Data Processing

Demultiplexing, filtering, and clustering of reads were performed with the inclusive pyRAD software pipeline (Eaton and Ree, 2013). After assigning reads to individuals based on a 6-bp barcode, bases with Phred quality scores less than 20 were recorded as missing (Ewing et al., 1998; Ewing and Green, 1998), and reads with greater than 10 missing sites were discarded.
Filtered reads with 85% sequence similarity or greater were then clustered within samples, using the USEARCH algorithm (Edgar, 2010) implemented in *pyRAD* to group reads into contigs based on percent sequence similarity. Error rate and heterozygosity were then estimated and used to create consensus sequences for each cluster. Consensus sequences were clustered at 85% similarity across samples and aligned with MUSCLE v3.8.31 (Edgar, 2004), and final loci with a total depth greater than 8 were extracted. For most individuals, data were only collected for a fraction of the total loci (see results, Table 1). Additional custom Python scripts were used to divide the final data set into seven partitions, some of which were eventually combined, in order to explore the effect of missing data (Table 2).

**Phylogenetic Methods and Partitioning by Missing Data**

Sequence data from each partition were concatenated and analyzed in RAxML v.8.0.19 under the GTRGAMMAI model with 100 fast bootstrap replicates (Stamatakis, 2014; Stamatakis et al., 2008). The resulting trees were compared using the Tree Farm package in MESQUITE (Maddison and Maddison, 2011; Maddison et al., 2011). Although the number of loci recovered increased as the minimum number of individuals required to define a locus was decreased (Table 2), partitions 1-6 produced maximum likelihood (ML) trees that were largely in agreement and well-supported (Table 2, Table 3). Only partition 7, in which nearly 95% of total data was missing, recovered a relatively inconsistent
Table 2. Loci divided into partitions based on the number of taxa missing data.

<table>
<thead>
<tr>
<th>Partition</th>
<th>Taxa without data</th>
<th>Loci</th>
<th>Characters</th>
<th>Missing characters (%)</th>
<th>Mean ML Bootstrap</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-19</td>
<td>175</td>
<td>25287</td>
<td>24.3</td>
<td>93.9</td>
</tr>
<tr>
<td>2</td>
<td>20-29</td>
<td>337</td>
<td>48574</td>
<td>39.6</td>
<td>94.2</td>
</tr>
<tr>
<td>3</td>
<td>30-39</td>
<td>746</td>
<td>107926</td>
<td>55.0</td>
<td>92.3</td>
</tr>
<tr>
<td>4</td>
<td>40-49</td>
<td>1880</td>
<td>272394</td>
<td>69.9</td>
<td>94.8</td>
</tr>
<tr>
<td>5</td>
<td>50-56</td>
<td>4142</td>
<td>596665</td>
<td>81.9</td>
<td>93.9</td>
</tr>
<tr>
<td>6</td>
<td>57-60</td>
<td>5248</td>
<td>750929</td>
<td>89.0</td>
<td>91.8</td>
</tr>
<tr>
<td>7</td>
<td>61-63</td>
<td>48225</td>
<td>6904713</td>
<td>94.8</td>
<td>66.7</td>
</tr>
</tbody>
</table>

Table 3: Comparisons of ML phylogenetic trees based on unique data partitions. Each cell contains the proportion of shared clades and the patristic distance correlation between trees generated from the numbered partitions, separated by "/".

<table>
<thead>
<tr>
<th></th>
<th>1/1</th>
<th>0.79 / 0.98</th>
<th>0.83 / 0.98</th>
<th>0.86 / 0.98</th>
<th>0.86 / 0.98</th>
<th>0.75 / 0.96</th>
<th>0.38 / 0.32</th>
<th>1/1</th>
<th>0.81 / 0.97</th>
<th>0.81 / 0.98</th>
<th>0.78 / 0.97</th>
<th>0.75 / 0.96</th>
<th>0.40 / 0.33</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.86 / 0.98</td>
<td>0.86 / 0.98</td>
<td>0.83 / 0.97</td>
<td>0.79 / 0.95</td>
<td>0.40 / 0.31</td>
<td></td>
<td>1/1</td>
<td>0.89 / 0.98</td>
<td>0.84 / 0.98</td>
<td>0.79 / 0.96</td>
<td>0.40 / 0.34</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/1</td>
<td>0.84 / 0.98</td>
<td>0.81 / 0.98</td>
<td>0.79 / 0.96</td>
<td>0.40 / 0.34</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
<td>1/1</td>
<td></td>
<td></td>
<td></td>
<td>0.41 / 0.36</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

and poorly supported topology. Subsequently, the 12,528 loci from partitions 1-6 were concatenated, totaling 1.75 million base pairs (79.5% missing data). An ML analysis was then performed on this total-evidence data set in RAxML under the GTRGAMMAI model.

Initial tree searches were performed on all sequenced taxa in order to confirm monophyly of species duplicates. Subsequently, to speed computation, duplicates with more missing data were removed. The topologies of four samples with high levels of missing data were inconsistent and poorly supported among
partitions, so these samples were also removed, resulting in a final data set comprising 66 taxa and 61 species (Table 1).

Bayesian analysis was performed in BEAST v. 2.1.1 under a GTR+I+\(\Gamma\) substitution model, a relaxed lognormal clock, and a birth-death model (Bouckaert et al. 2014). The age of the common ancestor of *Adelpha* and *Limenitis* was calibrated with a prior normal distribution (\(\mu = 12.5\) 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). Due to constraints in program memory, all 12,528 concatenated loci could not be analyzed simultaneously. To compensate, independent searches of at least 7.6 million generations, sampling every 3,000 generations, were performed on twelve sets of 1,000 concatenated loci randomly selected from the total dataset. After determining convergence in Tracer v1.6.0 (Rambaut et al., 2013), the last 5 million generations of each run were combined with LogCombiner v2.1.1 (Rambaut and Drummond, 2014). TreeAnnotator v2.0.3 (Rambaut and Drummond, 2013) was used to determine the consensus tree, with posterior probability support, from the total, combined distribution of 8,042 trees.

Previous multi-locus phylogenetic studies have acknowledged that concatenation methods may result in spuriously high bootstrap support (Gadagkar et al., 2005; Wagner et al., 2013). Genomic regions sampled by RAD may vary by mutation rate, base composition, transition/transversion ratio, selection pressure, and other factors that are ignored when concatenated sequences are analyzed under
a single model (Rannala and Yang, 2008). Adding greater amounts of data to the analysis reduces sampling error, leading to higher bootstrap support, but does not relieve these systematic biases (Rannala and Yang, 2008; Kumar et al., 2012; Rubin et al., 2012). To assess the scope of this issue, we employed three alternative approaches to phylogeny estimation. First, 223,169 binary SNPs, present in at least 10% of individuals and with minor allele frequency ≥ 0.05, were identified from the total raw data set and used to estimate a species tree in RAxML under the ASC_GTRGAMMA model, which accounts for the ascertainment bias inherent in selecting only variable sites. Second, the 12,528 loci were scored for presence or absence of data for each individual, and this presence-absence matrix was analyzed in RAxML under the BINGAMMAI model. Finally, a species tree was estimated using a set of 12,528 individual gene trees withNJst on the STRAW server (Liu and Yu, 2011; Shaw et al., 2013). NJst calculates the average number of internodes between all pairs of species across the unrooted input gene trees, which were themselves generated here with PHYML v3.0 under the HKY substitution model (Guindon et al. 2010; see DaCosta and Sorenson, in review, for details). Such "species tree" methods are advantageous because they synthesize data from gene trees with independently estimated parameters, eliminating the bias stemming from applying the same model to all concatenated loci (Ranalla and Yang, 2008).
**Character Evolution**

Host plant usage data was primarily collected from Willmott (2003a), Scott (1986), and HOST, an online global database of Lepidopteran host plants maintained by the Natural History Museum, London (Robinson et al., 2010). Photographs and species classifications of the nine putatively mimetic *Adelpa* wing patterns were also collected from Willmott (2003a) (Fig. 1). Categorical host plant and wing pattern characters were mapped on the consensus likelihood and Bayesian trees with MESQUITE, and ancestral states along the tree were determined with parsimony (Maddison and Maddison, 2011).

**Diversification Rate Analyses**

Several tests of diversification rate heterogeneity were applied to the consensus Bayesian tree. First, SymmeTREE v1.1 was used to perform seven tests of whole-tree variation in diversification rate (Chan and Moore, 2005). These tests consider only tree topology and cannot account for incomplete taxon sampling. Therefore, current phylogenetic results were combined with polytomous topologies of the missing taxa based on previous studies of morphological traits and several genes (Fig 2; Willmott, 2003b; Tuzov et al., 2000; Mullen et al., 2006). To address uncertainty in the total topology, 100,000 random resolutions were generated under a taxon-size-sensitive equal-rates Markov model, and both the completely and incompletely sampled trees were tested for rate heterogeneity. Additionally,
SymmeTREE was used to test each branch as the location of a shift in diversification rate by comparing observed branching patterns to those expected under an equal-rates model.

Based on these results, the tree was divided into two clades hypothesized to have experienced different diversification rates. BayesRate v1.4 (Silvestro et al., 2011) was used to estimate clade-specific rates from a random sample of 500 Bayesian trees under a two-rate, pure-birth model with a uniform diversification prior. A search of 300,000 generations was performed on each tree, sampling every 2,500 generations after 30,000 generations of burn-in.

In addition to determining clade-specific rates under a two-rate model, we used two methods to compare the marginal likelihoods of models with varying numbers of rates. In BayesRate, we compared models estimating one rate or two rates across the tree (i.e., no rate shifts or one rate shift) using thermodynamic integration. Finally, in BAMM v2.0 (Rabosky, 2014), we implemented an exponential change function for 500,000 generations to determine the most likely number of rate shifts across the tree. Both of these methods can account for incomplete sampling when the proportion of sampled taxa is specified.
RESULTS

Sequencing

A total of 230.4 million reads were generated, of which 156.9 million passed stringent quality filters. Each individual retained an average of 1.33 million reads (range: 0.56-3.81 million; Table 1). Clustering within samples produced an average of 7,468 loci per individual (range: 597-15,008; Table 1). There was no relationship between number of reads and number of loci ($R^2 = 0.0074$), suggesting that sampling depth was sufficient to recover all ddRAD loci in most individuals.

In general, most loci were highly variable across the phylogenetic breadth of taxa, with an average of 28.8 SNPs per 145-basepair locus. However, average heterozygosity within individuals was significantly higher in *Adelpha* than in *Limenitis* (0.0085 vs. 0.0060; $p < 0.0001$). Nucleotide diversity ($\pi$) among taxa was also strongly related to the number of taxa missing data for a given locus ($R^2 = 0.998$ for Partitions 1-6; Fig. 2). For example, the mean per-locus nucleotide diversity in Partition 6 was almost twice as high as the mean nucleotide diversity of loci in Partition 1.

Phylogenetics

The RAD dataset provides unparalleled resolution of species relationships within *Adelpha* and *Limenitis* (Fig. 3). Nearly all nodes in the ML tree have bootstrap support of 95 or greater. The few nodes with relatively low support all follow short
Figure 2. Missing data is strongly correlated with nucleotide diversity. Each point represents the average value of nucleotide diversity for loci within a partition branches, possibly due to the increased likelihood of incomplete lineage sorting over short divergence time scales (Fig. 3; Wiens, 2008; Rannala and Yang, 2008). Support is similarly high among trees produced from all other methods (Table 4; Fig. 4; Fig. 5).

The genus *Limenitis* is entirely embedded within the new world *Adelpha*, offering unilateral support for the paraphyly of the *A. alala* clade and the rest of the genus (Fig. 2). Previous *Adelpha* species groupings based on morphological similarity are largely consistent with our phylogenetic results, particularly within the *alala*, *serpa*, and *phylaca* groups (Willmott, 2003b). However, the finer resolution offered by genomic data suggests the broadening of the *iphiclus* group, as
Figure 3. Maximum likelihood tree based on concatenated sequence data for 12,528 loci. Nodes with bootstrap support ≥ 95 are unlabeled. Branches are colored by host plant family; for clarity, families that host fewer than two taxa are omitted. No host-plant information is known for taxa shown in gray. Species groupings are after Willmott, 2003a.
Table 4. Data type and average node support for phylogenetic models applied to RAD sequence data.

<table>
<thead>
<tr>
<th>Phylogenetic model</th>
<th>Data</th>
<th>Average Support</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTRGAMMA (RAxML)</td>
<td>12,528 concatenated loci</td>
<td>97.46</td>
</tr>
<tr>
<td>GTR+I, lognormal clock, birth-death (BEAST)</td>
<td>12 samples of 1000 concatenated loci</td>
<td>90.06</td>
</tr>
<tr>
<td>Njst (STRAW)</td>
<td>12,528 ML gene trees</td>
<td>94.33</td>
</tr>
<tr>
<td>ASCGTRGAMMA (RAxML)</td>
<td>223,169 binary SNPs</td>
<td>98.78</td>
</tr>
<tr>
<td>BINGAMMA (RAxML)</td>
<td>presence/absence of 12,528 loci</td>
<td>90.62</td>
</tr>
</tbody>
</table>

Table 5. Comparisons of phylogenetic trees based on different models and data types. Each cell contains the proportion of shared clades and the patristic distance correlation between trees, separated by "/".

<table>
<thead>
<tr>
<th></th>
<th>RAxML</th>
<th>BEAST</th>
<th>SNPs</th>
<th>P/A</th>
<th>NJst</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAxML</td>
<td>1 / 1</td>
<td>0.85 / 0.98</td>
<td>0.94 / 0.99</td>
<td>0.49 / 0.88</td>
<td>0.76 / 0.98</td>
</tr>
<tr>
<td>BEAST</td>
<td>1 / 1</td>
<td>1 / 1</td>
<td>0.85 / 0.98</td>
<td>0.49 / 0.86</td>
<td>0.74 / 0.95</td>
</tr>
<tr>
<td>SNPs</td>
<td></td>
<td></td>
<td>1 / 1</td>
<td>0.51 / 0.87</td>
<td>0.76 / 0.97</td>
</tr>
<tr>
<td>P/A</td>
<td></td>
<td></td>
<td></td>
<td>1 / 1</td>
<td>0.54 / 0.86</td>
</tr>
<tr>
<td>NJst</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 / 1</td>
</tr>
</tbody>
</table>

well as the splitting of the morphologically similar *cocala* group into two distinct lineages.

As in the partitioning analysis, trees constructed from most methods were well-supported and in strong agreement (Fig. 5; Table 5). The major topological differences occurred among the five most derived species of Eurasian *Limenitis* (Node K), frequently represented by poorly supported, short branches. While a few sister taxa appear uniquely monophyletic in one of the five trees (e.g, Node G, Fig. 3-4), only two species, *A. corcyra* and *L. sydyi*, appear in substantially different positions.

*L. sydyi* is basal to the Eurasian *Limenitis* in all ML trees, but sister to *L. populi*
**Figure 4.** Bayesian maximum clade credibility tree based on 12 independent samples of 1,000 concatenated loci. Nodes with posterior probabilities $\geq 95$ are unlabeled. Branches are colored by mimetic *Adelpha* wing pattern type. Taxa in gray are not involved in *Adelpha* mimicry.
Figure 5. 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.
in the NJst and BEAST trees. The uncertainty in the placement of *A. corcyra* in relation to other members of the *alala* group, however, is likely due to its sparse sequence data; *A. corcyra* recovered the least data of all samples included in the tree (Table 1). As expected, missing data had the greatest effect on the tree constructed from a presence-absence matrix (Fig 5b). Three *Adelpha* and one *Limenitis* taxa, all sampled over a decade before library construction, were erroneously placed with the outgroups in the presence-absence tree (Fig. 5b). DNA degradation over time may have contributed to random loss of restriction sites, as evidenced by the small number of loci recovered within these individuals (Table 1).

*Character Evolution*

Host plant specialization is quite labile over time, changing states 27 times in the history of the ingroup (Fig. 3). The ancestral feeding state for *Limenitis* and the *A. alala* group is Caprifoliaceae, although the North American *Limenitis* shifted to Salicaceae (Fig. 3). The ancestral feeding state for the lowland *Adelpha* is Rubiaceae. Although we have poor sampling of Amazonian species in the *A. serpa* group, it appears that Rubiaceae specialization was preceded by exceptional polyphagy in *A. serpa* at the base of the clade (Fig. 3, Branch 0).

*Adelpha* wing patterns are also highly labile, changing states 24 times among the 46 taxa in the tree (Fig. 4). 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 retained as the ancestral state in most of the large *Adelpha* clade.

Nonetheless, the *alala* pattern reappears five times in the lowland clade, separated from the *alala* group by nearly 12 million years of evolution (Fig. 4, Node A). In fact, all patterns for which we have sampled more than one representative species, including those indicated by green and purple, appear to have multiple independent origins in the lowland *Adelpha*.

**Diversification Rates**

Bayesian age estimates of several nodes (Table 6) allow for the comparison of simple diversification rates across groups. Uncertainty in these age estimates is indicated by the 95% highest posterior density intervals (95% HPD), the smallest interval that contains 95% of the density of the Bayesian posterior distribution. For example, the crown age of *Adelpha* and *Limenitis* was estimated at 11.94 million years (95% HPD 8.00-15.26 mya; Node A), close to previous estimates of 12.5 and 13.4 my (Mullen et al., 2011; Wahlberg et al., 2009). This translates to an overall diversification rate for *Adelpha* and *Limenitis* of 8.88

<table>
<thead>
<tr>
<th>Node</th>
<th>Estimated Age (my)</th>
<th>95% HPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11.94</td>
<td>8.00 - 15.26</td>
</tr>
<tr>
<td>B</td>
<td>10.90</td>
<td>7.06 - 13.77</td>
</tr>
<tr>
<td>C</td>
<td>11.19</td>
<td>7.54 - 14.42</td>
</tr>
<tr>
<td>F</td>
<td>8.14</td>
<td>5.59 - 10.79</td>
</tr>
<tr>
<td>G</td>
<td>7.76</td>
<td>5.23 - 10.29</td>
</tr>
<tr>
<td>H</td>
<td>6.92</td>
<td>4.84 - 9.48</td>
</tr>
<tr>
<td>I</td>
<td>4.39</td>
<td>2.39 - 6.66</td>
</tr>
<tr>
<td>J</td>
<td>3.30</td>
<td>1.45 - 4.44</td>
</tr>
<tr>
<td>K</td>
<td>5.63</td>
<td>4.05 - 7.86</td>
</tr>
<tr>
<td>L</td>
<td>1.46</td>
<td>0.89 - 2.11</td>
</tr>
<tr>
<td>M</td>
<td>5.01</td>
<td>3.34 - 6.69</td>
</tr>
</tbody>
</table>

Table 6. Bayesian age estimates for labeled nodes.
species per million years. By comparison, the rate is 1.93 for *Limenitis*, 1.34 for the *A. alala* clade, and 7.06 for the lowland *Adelpha*.

Short backbone branch lengths in the large *Adelpha* clade (e.g., Branch I) indicate periods of rapid genetic differentiation, supported by strong statistical evidence for diversification rate heterogeneity across the tree. SymmeTREE rejected the hypothesis of an equal-rates Markov random branching model with all test statistics, for both the incompletely and completely sampled trees. Overall support for rate heterogeneity and tree imbalance was slightly weaker in the polytomous tree (Fig. 6, *p* = 0.001-0.01) than in the fully resolved Bayesian tree (Fig. 4, *p* = 0.0004 – 0.01).

When missing taxa were ignored, a single branch near the base of the lowland *Adelpha*, immediately after the branching of *A. melona leucocoma*, was supported as the location of a diversification rate shift (Fig. 3, Branch F; *Δ*₁: *p* = 0.033; *Δ*₂: *p* = 0.042). When missing taxa were included in the analysis, statistical support was less strong for a nearby branch, immediately preceding the branching of *A. melona leucocoma* (Branch D; *Δ*₁: *p* = 0.112; *Δ*₂: *p* = 0.117). However, this branch was the most probable location of a rate shift among the 185 total branches. Subsequently, Node E (Fig. 3) was used to divide the tree into two clades to evaluate clade-specific rates. Under a two-rate, pure-birth model, the mean estimated speciation rate was significantly higher for "post-shift" taxa than "pre-shift" taxa (0.308 vs. 0.251; effective sample sizes = 863 and 1115; two-tailed *p* < 0.0001).
Figure 6. Estimate of all species relationships in *Adelpha* and *Limenitis*, including species not sampled for molecular analysis. Topological uncertainty is indicated by polytomies.

However, despite the above evidence for rate shifts and rate heterogeneity, Bayesian model comparisons did not promote models with greater than one diversification rate. Thermodynamic integration in BayesRate preferred a single-rate model over a two-rate model ($2\ln(B) = 8.96$; Kass and Raftery, 1995), while BAMM was unable to distinguish between the likelihoods of models with zero or one rate shifts ($2\ln(B) = 1.98$).
DISCUSSION

Phylogenetics

Sequenced RAD tags from *Adelpha* and *Limenitis* provided a wealth of reliable information across all levels of evolutionary divergence. Although allelic dropout, here resulting from mutations in restriction sites over timescales of millions of years, has been shown to contribute substantial error into population genetic analyses (e.g. Miller et al., 2002; Pompanon et al., 2005; Arnold et al., 2013), we show that patterns of missing data carry a true phylogenetic signal. The ML tree constructed from presence/absence data was in strong agreement with all other trees (Fig. 5b), as were trees constructed from loci partitioned by the number of taxa missing data (Table 3). Our results suggest that while some threshold for a maximum level of missing data is required, it may be overly conservative to retain only loci that appear in all or most individuals. Similarly, trees constructed from concatenated data, SNPs, and gene trees produced very similar results, despite the strong qualifications used with concatenation methods in previous studies (e.g., Wagner et al., 2013). Our results, which corroborate recent findings in *Drosophila* and parasitic finches (Cariou et al., 2013; DaCosta and Sorenson, *in review*), suggest that many modern methods may be adequate for the analysis of RAD sequence data. Given that as few as 175 well-sampled, concatenated loci can provide a largely accurate phylogenetic tree (Table 3), future phylogenetic and diversification studies
may benefit from focusing on the collection of sequence and phenotypic data from more taxa, rather than more genetic loci per taxon or more variation in methods of analysis.

*Extraordinary Diversification Rates*

The results of our diversification rate analyses are consistent with previous findings of a rate shift among lowland *Adelpha* (Mullen et al. 2011), but produced mixed inferences depending on the model implementation. Topological analyses in SymmeTREE, for example, indicated that the branches surrounding the divergence of *A. melona leococoma* represent an area where the observed branching patterns are more extreme than expected under an equal-rates model (Fig. 3., Branches D, F). In addition, a two-rate model implemented in BayesRate found significantly different diversification rates between the monophyletic group including *A. melona leucocoma* and the other Adelpha and Limenitis taxa. However, additional model comparison tests performed in BayesRate and BAMM, which account for the proportion of unsampled taxa, found no evidence for more than one rate of diversification across the tree. One interpretation of this result is that intragroup phylogenetic uncertainty may reduce power and increase variance of statistical estimates of diversification rate change (Peña and Espeland, 2013). Therefore, more complete taxon sampling will likely be necessary to confidently localize rate shifts in future studies.
Instead, by considering all of *Adelpha* and *Limenitis* a single adaptive radiation, we can estimate the overall diversification rate of clades across the tree based on our Bayesian divergence time estimates. For example, all 21 described *Limenitis* species (Tozov et al., 2000; Mullen et al., 2006) and 85 *Adelpha* species (Willmott, 2003a) are descended from node A (Fig. 3, Table 6), implying an extraordinary total diversification rate of 8.88 new species every million years. This rate is twice as fast as the most rapid insect radiation known—4.17 species per million years in Hawaiian *Laupala* crickets (Mendelson and Shaw, 2005)—and over fifty times faster than the average estimated rate of arthropod diversification, which is only 0.16 species per million years (Coyne and Orr, 2004).

*Host plant shifts and mimicry: ecological adaptations*

Reconstruction of the history of character evolution on the phylogeny indicates that host plant use among *Adelpha* and *Limenitis* is both diverse and highly labile (Fig. 3). We find evidence that, following the origin of extreme polyphagy in *A. serpa*, the lowland *Adelpha* shifted to Rubiaceae-specialization ~9.5 mya (Fig. 3, Node E). This phenotype has been retained as the most parsimonious ancestral state for most nodes and taxa across the genus, although at least two smaller host shifts have also occurred in the *phylaca* and *cocala* groups (Fig. 3). In addition, although the *alala* species group and Eurasian *Limenitis* all feed on plants in the family
Caprifoliaceae, a recent host shift to Salicaceae has occurred among North American Limenitis (Fig. 3).

The widespread use of Rubiaceous plants in Adelpha is of particular interest, given the well-known production of anti-herbivorous, bioactive compounds by members of this plant family. Prior to the existence of a strong phylogenetic hypothesis for Adelpa, Aiello (1984) tentatively proposed that species whose larvae feed on Rubiaceae might form the unpalatable models for other Adelpha species, and subsequent work has shown that over three quarters of Rubiaceae in Panama produce alkaloid compounds known to repel herbivores (Soto-Sabenis et al., 2001; Kessler and Baldwin, 2002; Schmeller and Wink, 1998). Consistent with the hypothesis of chemical defense, each Rubiaceae-specialist Adelpha sampled in our study feeds on a genus known for harboring chemicals with poisonous, narcotic, or medicinal effects (Schultes, 1985; Quattrocchi, 2012; Soto-Sabenis et al., 2001). Therefore, it seems likely that a host-plant shift to Rubiaceae required physiological adaptation to anti-herbivorous toxins. Given that the first known Rubiaceae-specialist, *A. melona leucocoma*, is also at the center of estimates of an increase in diversification rate, it is possible that adaptation to a novel hosts may have played an important role in the incredibly rapid diversification of these butterflies.

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 & Brower 1971; Ritland 1991; Mullen et al. 2008), attempts to directly demonstrate
unpalatability in *Adelpha* have produced conflicting and inconclusive results (Prudic et al., 2002; Srygley and Chai, 1990; Pinheiro, 1996). Examination of the recovered phylogeny, however, clearly indicates that distinct wing patterns have multiple, independent origins across the tree, strongly implying that *Adelpha* wing patterns are adaptive and mimetic (e.g., Müller, 1879; Mallet and Gilbert, 1995; Ruxton et al., 2004; Mullen, 2006).

**Conclusion**

Examples of adaptive radiation, which are characterized by rapidly diversifying lineages that display enormous phenotypic and ecological variation, present some of the best opportunities to understand how microevolutionary processes acting within populations give rise to diversity across macroevolutionary timescales. However, identifying the proximate mechanisms promoting rapid diversification requires detailed knowledge of the evolutionary relationships among species. Our results demonstrate the utility of genome-wide RAD markers to fully resolve 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 extraordinarily rapid adaptive diversification in this group.


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Education

Masters of Arts  September 2014
Boston University
Biology - Ecology, Behavior, and Evolution
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Courses: Speciation, Population Genetics, Applied Genetic Analysis, Theoretical Evolutionary Ecology, Python

Bachelor of Science  December 2011
University of Oregon - Robert D. Clark Honors College
Biology major, Chemistry minor
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GPA: 4.07 on a 4.0 scale (Biology, 4.11)
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Research Experience

Masters Student, Sean Mullen Lab, Boston University  September 2012 - Present
-Phylogenetics, neotropical diversity, genome-wide association, RAD library preparation and sequencing

Adjunct Research Assistant, Patrick Phillips Lab, Institute of Ecology and Evolution, University of Oregon  September 2011-July 2012
-Evolutionary genetics of sexual conflict, inbreeding depression, and sex ratio
Undergraduate Researcher, Patrick Phillips Lab, February 2010-August 2011
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-Natural variation in sexual conflict; genetic mapping

Grants and Scholarships

NSF Graduate Research Fellowship 2013-2016
Boston University Dean’s Fellowship 2012-2013
University of Oregon Presidential Scholarship 2008-2011
National Merit Scholar 2008

Honors and Awards

Aaron Novick Award for a Distinguished Thesis in Science June 2012
Undergraduate Honors Thesis with Distinction November 2011
Biology Departmental Honors December 2011
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Publications and Presentations

Ecological specialization drives rapid diversification in Neotropical Adelpha butterflies.


Teaching Experience and Outreach

**Teacher**, BioBUGS, Boston University                      2014
**Instructor**, Undergraduate Chemistry Writing Program, Boston University 2013
**Volunteer**, Teen Overnight, Museum of Science (Boston) 2013
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