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Wing pattern evolution and the origins of mimicry among North American admiral butterflies (Nymphalidae: *Limenitis*)

Sean P. Mullen

Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14850, USA
Department of Biology, University of Maryland, College Park, College Park, MD 20742, USA

Abstract

The evolution of wing pattern diversity in butterflies has emerged as a model system for understanding the origins and maintenance of adaptive phenotypic novelty. Admiral butterflies (genus *Limenitis*) are an attractive system for studying wing pattern diversity because mimicry is common among the North American species and hybrid zones occur wherever mimetic and non-mimetic wing pattern races meet. However, the utility of this system has been limited because the evolutionary relationships among these butterflies remain unclear. Here I present a robust species-level phylogeny of *Limenitis* based on 1911 bp of two mitochondrial genes (COI and COII) and 904 bp of EF1-α for all five of the Nearctic species/wing pattern races, the majority of the Palearctic species, and three outgroup genera; *Athyma*, *Moduza* (Limenitidini), and *Neptis* (Limenitidinae: Neptini). Maximum-likelihood and Bayesian analyses indicate that the North American species are a well-supported, monophyletic lineage that is most closely related to the widespread, Palearctic, Poplar admiral (*L. populi*). Within North America, the Viceroy (*L. archippus*) is the basal lineage while the relationships among the remaining species are not well resolved. A combined maximum-likelihood analysis, however, indicates that the two western North America species (*L. lorquini* and *L. weidemeyerii*) are sister taxa and closely related to the wing pattern subspecies of the polytypic *Limenitis arthemis* species complex. These results are consistent with (1) an ancestral host-shift to Salicaceae by the common ancestor of the Poplar admiral and the Nearctic admiral lineage, (2) a single colonization of the Nearctic, and (3) a subsequent radiation of the North American forms leading to at least three independent origins of mimicry.
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1. Introduction

Phylogenetic studies of taxa that exhibit adaptive phenotypic variation provide valuable insights into the evolutionary mechanisms driving the origins of biodiversity. Historically, species of Lepidoptera have been favored subjects for ecologists and evolutionary biologists interested in the origin and maintenance of such variation (e.g., Ford, 1964; Goldsmidt, 1934; Nijhout, 1991; Sheppard, 1961) and early natural historians clearly recognized the importance of traits, such as wing pattern, in the process of speciation

(Bates, 1862). Indeed, the evolution of wing pattern diversity in butterflies has emerged as an important model system for studies of speciation, mimicry, and (most recently) the interface between evolution and development (McMillan et al., 2002; Reed and Serfas, 2004).

Admiral butterflies (genus *Limenitis*) are a particularly appealing system with which to address questions regarding wing pattern evolution and speciation. This genus is unusual among temperate butterflies in that mimicry has evolved multiple times and hybridization is frequent between wing pattern forms (Gunder, 1932; Platt and Brower, 1968; Poulton, 1909; Porter, 1989, 1990; Remington, 1958) (Fig. 1). Early work on this group produced one of the first experimental demonstrations of mimicry in
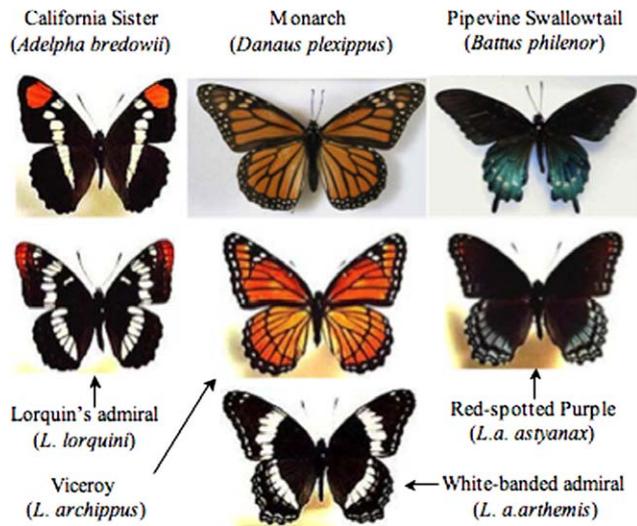
E-mail address: spm23@umd.edu.

Fig. 1. Wing pattern mimicry among North American Admirals (genus *Limenitis*) and their respective models.

butterflies (Brower, 1958) and contributed to the debate over how to define species boundaries in the face of hybridization (Fisher, 1958; Platt and Brower, 1968; Remington, 1968). More recently, work on the Viceroy butterfly (*L. archippus*) has led to increased awareness of the palatability spectrum and the subjectivity of traditional labels such as Batesian vs. Mullerian mimicry (Ritland, 1998; Ritland and Brower, 2000). Unfortunately, the utility of this group of butterflies as a subject of speciation research has been limited by the lack of a well-supported phylogenetic hypothesis.

An interesting facet of wing pattern evolution within the genus *Limenitis* is the apparent disparity between the largely stereotypic Palearctic fauna and the highly variable Nearctic forms. The Palearctic forms have a uniformly dark brown or black background wing color with one major transverse band of white pigment running across both the dorsal and ventral surfaces of the fore- and hindwings. Variability among these species is predominantly in the subtle variation of this white-band wing pattern element. In contrast, wing patterns in the Nearctic *Limenitis* are diverse. Three lineages of mimetic butterflies occur in North America, and the evolution of mimicry may have played a large role in the diversification of this group (Platt, 1983).

The most well-recognized example of mimicry among North American *Limenitis* involves the relationship between the Viceroy butterfly (*L. archippus*) and the chemically defended Monarch (*Danaus plexippus*). While initially considered a classic example of Batesian mimicry, subsequent work (Ritland, 1998; Ritland and Brower, 1991) has demonstrated that the palatability of Viceroy populations varies considerably across its range. In fact, in parts of its range, the Viceroy is less palatable than its model and the relationship between the Monarch and Viceroy is perhaps more accurately viewed as Müllerian in these areas. Another example of mimicry in the genus *Limenitis* occurs in the western United States where Batesian mimicry has

evolved between the Lorquin's admiral (*L. lorquini*) and the California Sister butterfly (*Adelpha bredowii*) (Prudic et al., 2002) (Fig. 1). While *Adelpha bredowii* is only moderately unpalatable to captive Scrub Jays (*Aphelocoma californica*), this species clearly serves as the model for the fully palatable Lorquin's admiral where the two species co-occur. A final example of mimicry among Nearctic *Limenitis* involves populations of mimetic admirals belonging to the polytypic *Limenitis arthemis* species complex found in the southeastern and southwestern United States. Each of these subspecies, *L. a. astyanax* in the southeast and *L. a. arizonensis* in the southwest, is considered a Batesian mimic of the Pipevine Swallowtail (*Battus philenor*) (Platt et al., 1971). The Pipevine swallowtail, which feeds on species of *Aristolochia*, is continuously distributed throughout the southern United States from the mid-Atlantic region of the east coast as far west as California. In contrast, mimetic populations of *Limenitis arthemis* have a disjunct distribution and do not occur across much of central Texas due to an absence of suitable host plant (Scott, 1986). North of the range of the model (*Battus*) populations of *L. arthemis* are non-mimetic and, like most of the Palearctic forms, have large, transverse white bands across the central portions of both the fore- and hindwings. This subspecies (*L. arthemis arthemis*) is distributed across the northeastern United States and Great Lakes region westward across much of Canada to Alaska. A broad parapatric hybrid zone between non-mimetic, white-banded populations of *L. a. arthemis* and mimetic red-spotted purples (*L. a. astyanax*) occurs across central New England and the Midwest and individuals with intermediate phenotypes are commonly produced.

Resolution of the causes of the dramatic differences in wing pattern diversity between Nearctic and Palearctic forms requires a clear understanding of the relationships within the genus and of the origins of the North American species. Whether a single colonization event or repeated episodes of migration from the Palearctic are responsible the current assemblage of North American forms influences how we interpret the role that mimicry has played in the Nearctic radiation of *Limenitis* butterflies. In the former case, we expect the North American species to be a monophyletic lineage and, if so, the evolution of mimicry must have been strongly associated with, or even a driver of, speciation in this group. A history of multiple colonizations would suggest that speciation preceded the origins of novel, mimetic wing patterns among the Nearctic taxa. Under this scenario, reproductive isolation between lineages would facilitate the fixation of different mimetic patterns and, therefore, wing pattern novelty might be best viewed as a result of speciation rather than a potential driver of this process.

Contrasting patterns of larval host plant use in Eurasian vs. North American admirals suggest that a single colonization may best explain the origins of the North American forms. For instance, most Palearctic species of *Limenitis* feed on different species of honeysuckle (*Lonicera* spp.). The

exception is the Poplar admiral (*L. populi*) that, unlike its Old World congeners, feeds exclusively on aspen (Salicaceae: *Populus tremulus*) (Georgiev and Beshkov, 2000). All of the North American *Limenitis* feed on Salicaceae as well. This suggests that an ancestral host plant shift could have facilitated the colonization of the Nearctic. However, without knowledge of the phylogenetic relationships between the two continental faunas of these butterflies, it is difficult to assess this hypothesis. Finally, it is currently unclear whether the wing pattern diversity among the North American *Limenitis* simply represents geographical variation within one, or several, highly variable species (see Porter, 1989, 1990) or if the phenotypic diversity observed among these populations reflects a deeper history of population differentiation and/or speciation.

This paper presents a robust molecular phylogeny for *Limenitis* based on two mitochondrial genes (COI and COII) and the single-copy nuclear gene Elongation Factor 1-α (EF1-α). I show that the Nearctic *Limenitis* represent a well-supported monophyletic lineage and that each wing pattern race represents a discrete mitochondrial lineage within this larger clade. Furthermore, I demonstrate that the Poplar admiral (*L. populi*) is the closest extant Palearctic relative of the North American forms and argue that an

ancestral host plant switch to tree species in the plant family Salicaceae has played a significant role in the colonization of North America and subsequent radiation of the Nearctic forms. Finally, my results suggest that the disparity in wing pattern diversity between Nearctic and Palearctic *Limenitis* may be due to increased opportunities for the origins of wing pattern mimicry driven by close associations between North American admiral butterflies and northern exemplars of unpalatable, South American butterfly genera.

2. Materials and methods

2.1. Taxon sampling

Fifteen species of *Limenitis* (subtribe: Limenitidini) were sampled, including all of the North American and European species and most of the east-Asian taxa (Table 1). To explore the relationships and species boundaries among the North American taxa, multiple specimens representing the geographic range of each species were included. Several Palearctic species (*L. cleophas*, *L. dubernardi*, and *L. ciocolatina*) with geographic ranges restricted to northwestern China, as well as one Indo-Pacific endemic (*L. imitata*),

Table 1
Collection information for *Limenitis* specimens and outgroup taxa used in phylogenetic analysis

Species name	Voucher #	Collecting locality	Collection date	Collected by	Accession No.
L. archippus	SPM001-003	New York, USA	05/01	S. Mullen	DQ205122
		Florida, USA	03/02	S. Mullen	DQ205114
		Alberta, CAN	07/02	B. Beck	DQ205109
	SPM043	Nebraska, USA			DQ205128
L. lorquini	SPM004-005	Oregon, USA	08/01	R. Worth	DQ205106
		British Columbia, CAN	06/02		DQ205132
L. wiedemeyeri	SPM006-007	Colorado, USA	07/01	Mark Walker	DQ205101
L. a. arthemis	SPM008-012	Alaska, USA	08/01	S. Murphy	DQ205125
		Alberta, CAN	08/02	G. Anweiler	DQ205129
		Manitoba, CAN	07/02	A. Foster	DQ205108
		Quebec, CAN	07/03	S. Mullen	DQ205121
		Maine, USA	06/02	A. Grkovich	DQ205124
L. a. astyanax	SPM013-017	Kentucky, USA	08/01	L. Koehn	DQ205130
		South Carolina, USA	06/02	S. Mullen	DQ205131
		Arkansas, USA	08/01	S. Mullen	DQ205112
		Mississippi, USA	05/03	R. Patterson	DQ205107
		Virginia, USA	06/02	S. Mullen	DQ205113
L. a. arizonensis	SPM018-022	Arizona, USA (5)	06/01–07/03	K. Dyer, M. Walker	DQ2051010/17/20/23/26
L. amphyssa	SPM023	S.E. Siberia	07/02	A. Chichvarkin	DQ205104
L. moltrechti	SPM024	S.E. Siberia	06/02	A. Chichvarkin	DQ205127
L. helmanni	SPM025-026	S.E. Siberia	06/02	A. Chichvarkin	DQ205119
L. homeyeri	SPM027-028	S.E. Siberia	06/02	A. Chichvarkin	DQ205118
L. camilla	SPM029-030	France	007/01	N. Whalberg	DQ205111
L. populi	SPM031-032	Sweden	06/02	N. Whalberg	DQ205105
L. reducta	SPM033-034	France	06/02	N. Whalberg	Pending
L. glorifica	SPM035	Japan	06/02	A. Chichvarkin	DQ205115
L. doerriesi	SPM036-037	S.E. Russia	07/03	R. Yakovlev	DQ205116
L. staudingeri	SPM038	Seram Island	06/99	A. Chichvarkin	DQ205102
L. (Seokia) pratti	SPM039	S.E. Siberia	07/03	R. Yakovlev	DQ205103
Outgroups					
Moduza pintyana	SPM040	Philippines	07/02	A. Chichvarkin	DQ205134
Neptis speyeria	SPM041	S.E. Siberia	07/01	A. Chichvarkin	DQ205135
Athyma selenophora	SPM042	Hong Kong	06/02	A. Chichvarkin	DQ205133

were unavailable. It is unlikely that the inclusion of these taxa would significantly alter the recovered gene tree but future efforts will be made to include these taxa (Rosenberg and Kumar, 2001; but see Zwickl and Hillis, 2002). In addition, representatives of three outgroup genera were included, two from the subtribe to which *Limenitis* belongs (*Athyma*, *Moduza*) and one from the broader subfamily (*Neptis*, Limenitidinae: Neptini).

2.2. DNA amplification and sequencing

DNA was isolated from ~25 μg of flight muscle using a Qiagen (Valencia, CA) extraction kit. I sequenced two mitochondrial genes (COI, COII) and one nuclear gene (EF1-α). A 2.02 kb region of mtDNA, spanning a portion of cytochrome oxidase I (COI), tRNA leucine, and all of cytochrome oxidase II (COII) was amplified using universal insect mtDNA primers Ron (5′-TCCAATGCACTAA TCTGCCATATTA-3′) and Eva (5′-GGAGGATTTGG AAATTGATTAGTTCC-3′) (Willett et al., 1997). I also amplified a 1 kb fragment of EF1-α using the primers described by Monteiro and Pierce (2001). However, EF1-α sequence was only obtained from a subset of the sampled taxa due to difficulties associated with amplifying this gene from dried specimens. All PCRs (10 μl volume) contained 3 mM MgCl$_2$, 0.2 mM dNTPs, 50 mM KCl, 20 mM Tris (pH 8.4), 2.5 ng of each primer, and 1 U of *Taq* DNA polymerase (Gibco-BRL) and 1 μL of genomic DNA. PCR amplifications were performed using a thermal cycler (OmniGene, Hybaid) with the following amplification profile: 35 cycles of 60 s at 95 °C, 60 s at 50 °C, and 120 s at 72 °C. Successfully amplified products were cleaned for sequencing by incubating with 1 μl of Exonuclease I (20 U/μl) and Shrimp Akalaine Phosphatase (5 U/μl) (0.5 μl of each/sample) at 37 °C for at least 45 min. Exo/Sap reactions were terminated by a brief (10-min) incubation at 90 °C. The cleaned PCR fragments for both loci were sequenced directly on an ABI PRISM 377 automated sequencer using BigDye terminator labeling (Applied Biosystems). *Limenitis* specific primers were designed as new sequence data were acquired and used to sequence the internal regions of the amplified PCR fragment (Table 2). Contigs of overlapping sequences were constructed using Seqman (DNASTAR, Madison, WI) and edited to create consensus files for each individual sampled. Ambiguous base calls for EF1-α sequences were interpreted as possible cases of heterozygosity and scored with the appropriate IUB code. These consensus sequences were then imported into Megalign version 5.05 (DNASTAR,

Table 2
List of *Limenitis* specific primers used for sequencing

Primer name	Primer sequence
Int-1	5′-CCAGAAGTTTAYATTTTAATTTTACC-3′
Int-2	5′-GGAGTTAAYTTAACYTTTTTYCC-3′
Int-3	5′-GGYAAAAAYGTTAAYTTAACTCC-3′
Int-4	5′-GAAGAACAAATAATTGAATTAATTTG-3′
Int-5	5′-CAAATTAATTCAATTATTTGTTCTTC-3′

Madison, WI) and aligned using a delayed CLUSTAL W algorithm. This is a weighted method of sequential multiple sequence alignment that imposes position specific gap penalties that improves gap handling (Higgens et al., 1996). Trimming during the editing and alignment process resulted in final aligned data sets of 1911 bp for the mitochondrial genes and 904 bp for EF1-α.

2.3. Phylogenetic analysis

Phylogenetic analyses were done using PAUP* 4.0b10 (Swofford, 2003) and MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). The final alignment was exported from Megalign (DNASTAR, Madison, WI) in nexus format to PAUP* and tree topology was initially explored using distance-based neighbor-joining methods. For each maximum-likelihood analysis, the most appropriate DNA substitution model was determined with Modeltest 3.6 (Posada and Crandall, 1998) based on likelihood parameter estimates generated in PAUP*. Modeltest selected a GTR + I + Γ model for the mitochondrial genes using the hierarchical likelihood ratio tests (hLRTs) and a TrNef + Γ model of DNA substitution for EF1-α. While there is some debate in the literature regarding the best method for choosing a model of sequence evolution (Minin et al., 2003; Posada and Buckley, 2004), in this case both hLRTs and the Akaike Information Criterion (AIC) approaches support the same model for each data set. Maximum-likelihood heuristic searches were carried using the following parameter values for the mitochondrial COI and COII genes: nst = 6, rmat = [R(A-C) = 4.9631, R(A-G) = 1.7175, R(A-T) = 3.9307, R(C-G) = 2.3969, R(C-T) = 47.8576, and R(G-T) = 1.0], rates = gamma, shape = 0.6285 pinvar = 0.5644, and empirical base frequencies. Similarly, the ML analysis of EF1-α was done with the following model parameters: nst = 6, basefreq = equal, rmat = [R(A-C) = 1.0, R(A-G) = 10.2706, R(A-T) = 1.0, R(C-G) = 1.0, R(C-T) = 3.2678, rates = gamma, shape = 0.0055, pinvar = 0.

Bayesian phylogenetic analysis of each data set was done using MrBayes 3.1.1 (Ronquist and Huelsenbeck, 2003). This program implements a Markov Chain Monte Carlo (MCMC) method to explore parameter space and estimates the posterior probabilities of each parameter. By using Metropolis coupling (MCMCMC) the program can simultaneously run several chains with different stepwise acceptance thresholds to more thoroughly explore parameter space and avoid becoming trapped in local valleys. The appropriate models of DNA substitution were specified in MrBayes using the general lset values (mtDNA: nst = 6, rates = invgamma; EF1-α: nst = 6, rates = gamma) and allowing the program to converge on the best estimates of these model parameters. Four chains, one cold and three heated incremental (0.2 temp), were employed for a series of MCMC searches of varying lengths (1 million, 5 million, and 10 million generations) sampled every 100 generations. Clade credibility values were highly consistent among runs and the average standard deviation of the split frequencies

at the end of 1 million generations for the mitochondrial data set was 0.00621 and 0.00083 for EF1-α. In addition, the average value for the assessed potential scale reduction factors (PSRFs) was 1.001 and 1.002, respectively, indicating convergence (but see Cummings et al., 2003).

All of the sampled trees from the Markov Chain simulation for each analysis were imported into PAUP*4.0b (Swofford, 2003) and the first 5000 sampled trees were discarded as burn-in. The posterior probabilities were determined by building the consensus topology from the remaining 5001 sampled trees. Nodal support for the mitochondrial and nuclear topologies was investigated further using 10,000 parsimony bootstrap replicates (full heuristic search, accelerated transformation, and gaps treated as missing).

A combined analysis using individuals represented by both data sets (mtDNA + EF1-α) was also carried out. For this analysis, the data were partitioned by locus (mitochondrial vs. nuclear) and the appropriate model of DNA substitution was applied to each region separately with a portion of sites invariant and gene specific gamma rates. The shape of the rate parameter, gamma value, substitution matrix, and statefreqs were unlinked and separately determined [unlink shape = (all); unlink revmat = (all); unlink statefreq = (all)]. The rate parameter was allowed to vary across partitions by setting the default ratepr (fixed) to prest applyto = all ratepr = variable. In addition, a site-specific rate model was explored by partitioning the combined data set into three gene regions (COI, COII, and EF1-α) and, for each gene, allowing the Bayesian MCMC simulation to estimate the substitution rate for each codon position [unlink revmat = (all); unlink statefreq = (all); prset applyto = (all) ratepr = variable]. In both cases, run lengths of 5 million generations were used.

3. Results

3.1. Phylogenetic relationships

In the mitochondrial phylogeny the North American admirals are a monophyletic group with both high nonparametric bootstrap support and posterior probabilities (Figs. 2A and B). Palearctic *Limenitis* are paraphyletic with respect to the Nearctic forms and the Poplar Admiral (*L. populi*) is the sister lineage to the Nearctic forms. The genus *Limenitis*, as currently defined, appears polyphyletic given the position of the Palearctic species *L. pratti* and *L. staudingeri*, which fall together with species from two of the outgroup genera (Fig. 2A). The remaining Eurasian species, with the exception of *L. reducta*, form a strongly supported monophyletic clade with high Bayesian posterior probabilities but low parsimony bootstrap support (Fig. 2B). Among the North American species, the Viceroy (*L. archippus*) is the basal lineage (Fig. 2A) and the two western species, Lorquin's (*L. lorquini*) and Weidemeyer's (*L. weidemeyerii*) admirals diverge before the eastern polytypic white-banded/red-spotted purple (*L. a. arthemix–astyanax*) spe-

cies complex. This topology, however, does not have strong posterior support in the Bayesian analysis (Fig. 2B), which recovers a basal polytomy, resulting in little resolution among the North American species. With one exception, each of the subspecies within the eastern complex is recovered as a monophyletic group. A red-spotted purple (*L. a. astyanax*) sampled from a population located near the hybrid zone (northern Virginia) is basal to all other White-banded Admirals (*L. a. arthemis*). Finally, both maximum-likelihood and Bayesian approaches indicate that *L. a. arizonensis*, the southwestern subspecies of this complex, is basal to the two eastern wing pattern races (Figs. 2A and B).

In contrast, the phylogenetic tree reconstructed using EF1-α sequences separates the Nearctic and Palearctic taxa into two reciprocally monophyletic clades (Figs. 3A and B). Nodal support is much weaker in this gene tree, however, relative to the mitochondrial genealogy. The relationships within the Nearctic and Palearctic forms are similar to those found using mtDNA for the reduced taxon sampling (*L. pratti* and *L. staudingeri* were excluded). The Poplar admiral is basal to the remaining Palearctic forms and the Viceroy (*L. archippus*), again, falls basal within the Nearctic lineage. The results from EF1-α also support an early splitting event between the southwestern populations of *L. a. arizonensis* and the two eastern wing pattern forms of the *L. a. arthemis* species complex.

The combined analysis recovers monophyly of the Nearctic taxa with extremely high posterior support (Figs. 4A and B) under both the gene-by-gene and site-specific models of DNA substitution. The maximum-likelihood tree places the Poplar admiral basal to the Palearctic taxa but the posterior probability for this topology is non-significant. Furthermore, the combined analysis recovers a sister-relationship between the two western species (*L. lorquini* and *L. weidemeyerii*) and indicates that these two butterflies represent the sister lineage to the eastern wing pattern complex (Fig. 4A). The consensus tree recovers a polytomy consisting of: (1) all the Nearctic taxa, (2) the Poplar admiral, and (3) all of the remaining Palearctic forms (Fig. 4B).

3.2. Sequence diversity and introgression

In general, sequence divergence between Nearctic and Palearctic taxa was high for both the mitochondrial and nuclear genes sampled (Table 3). Mitochondrial DNA divergence (uncorrected *p*-distance) within *Limenitis* ranged from 1.8 to 11.8%. The highest levels of divergence correspond to *p*-distances between *Limenitis pratti* and its congeners. However, the mitochondrial genealogy places *L. pratti* as the sister lineage to *Athyma* and, therefore, a more conservative estimate of maximum divergence in this group is 9.2% (see Table 3). Average pairwise sequence divergence between the Nearctic and Palearctic taxa was 8.4% and, surprisingly, mitochondrial divergence was also high among all of the North American species and wing pattern forms of the *L. a. arthemis* species complex. In fact,

sequence divergence within the *L. arthemis* species complex (1.8–2.6%) exceeded the observed divergence between the two western U.S. species, *L. lorquini* and *L. weidemeyerii* (2.0%). In addition, although hybridization is considered widespread among the Nearctic species, there is little evidence for mitochondrial introgression among nominal species of North American Admirals based on this sampling.

4. Discussion

4.1. Monophyly of North American Limenitis

Taken together, topologies for mtDNA and EF1-α strongly support the view that all of the Nearctic taxa are the product of a single colonization of the Nearctic, presumably via the Beringian land bridge. The mitochondrial tree topology also suggests that the Poplar admiral (*L. populi*) is the closest extant Palearctic relative. The Beringian land bridge connected northeastern Siberian with northwestern North America throughout the Tertiary until approximately 5 mya when rising sea levels led to the for-

mation of the Bering Strait (Marincovich and Gladenkov, 1999, 2001). The high latitudes of the Arctic were forested during much of the Tertiary but gave way slowly to increasing tundra in the late Pliocene (Matthews and Ovenden, 1990); the current circumarctic tundra belt was in place by 3 mya (Matthews, 1979).

Given the close tie between herbivorous insects and their host plants, it seems likely that the colonization of North America by Palearctic *Limenitis* must have occurred at some point prior to the closure of the Bering land bridge and the onset of cyclic Pleistocene glacial episodes. Indeed, recent biogeographic analysis of disjunction patterns suggest that many temperate forest plant groups originated and diversified in eastern Asia and subsequently dispersed via a North Atlantic route over the Beringia land bridge at several different points in the last 30 million years (Donoghue and Smith, 2004). Furthermore, patterns of molecular divergence between the Palearctic and Nearctic species of *Limenitis* are consistent with this historical scenario. The average uncorrected pairwise sequence divergence between the North American taxa and the Palearctic is 9.2%.
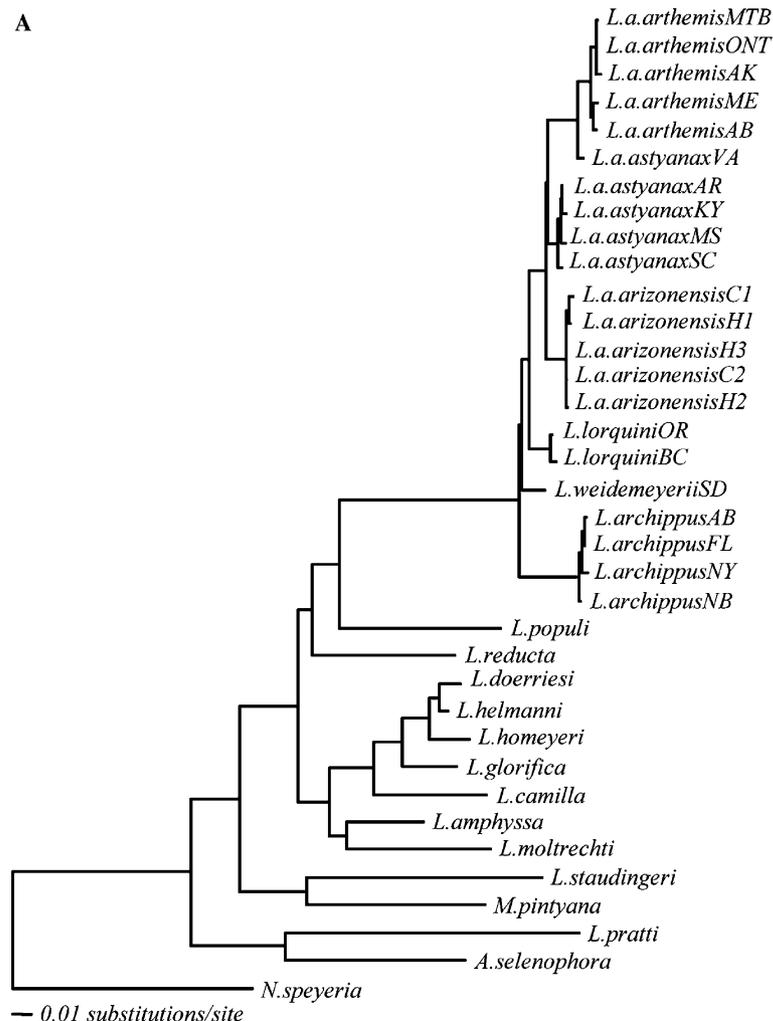

Fig. 2. (A) Maximum-likelihood phylogram of mitochondrial COI/COII for sampled representatives of Nearctic and Palearctic admiral species (*Limenitis*); tree is rooted using *Neptis speyeria* as outgroup. (B) Bayesian consensus tree from mtDNA COI/II showing relationships between the Nearctic and Palearctic species of *Limenitis*. Numbers above the branches represent posterior probabilities and those below are parsimony bootstrap values.
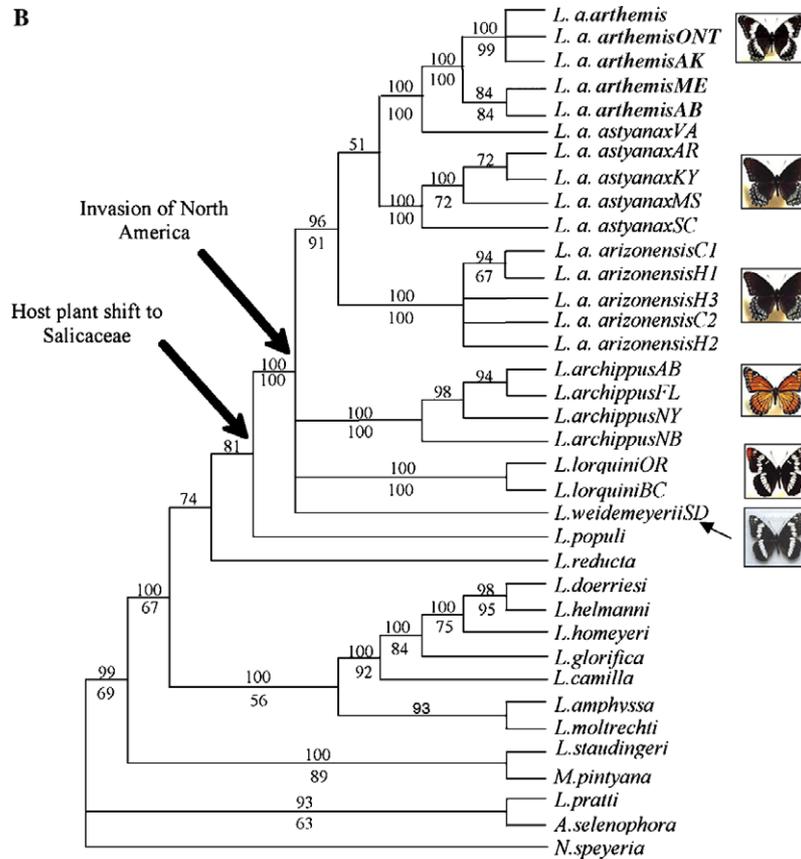
B

Fig. 2. (*continued*)

Assuming a crude divergence rate of 1.1–1.2% per lineage per million years (Brower, 1994), the colonization of the Nearctic dates to ∼4 mya, a date similar to the estimated closing of the North Atlantic colonization route. If this scenario is correct this suggests that biotic conditions between eastern Eurasia and the Western hemisphere are somehow fundamentally different and have led to an increased opportunity for the evolution of wing pattern novelty among the Nearctic forms. Interestingly, all three of the known models for admiral mimics in North America are northern exemplars of South America genera or, in the case of the Monarch butterfly (*Danaus plexippus*), are tied to warm Central American environs for at least part of their life cycle. For example, the California Sister belongs to the large South American genus *Adelpha* (Nymphalidae), well known for its rapid predator escape speed and for mimicry rings in the neotropics (Willmott, 2003). Thus, the relationship between unpalatable model butterflies from South America and North Amerian *Limenitis* suggests that faunal interactions between these two continents, possibly caused by climate-driven changes in the geographic ranges of northern taxa, have played an important role in wing pattern evolution and mimicry in this group. What remains unclear is whether the absence of wing pattern diversification in the Palearctic is due to an absence of unpalatable model species in this region or whether it is due to other constraints on wing pattern evolution.

4.2. Larval host plant use and biogeography

The phylogenetic position of the Poplar admiral (*L. populi*) as the closest extant relative of the Nearctic taxa is consistent with the hypothesis that host plant use has been important in the biogeographic history of the North American species. Host plant use in Palearctic *Limenitis*, much like wing pattern, shows little evidence of divergence. Most of the Palearctic species feed exclusively on species of Honeysuckle (*Lonicera* sp.) (Gorbunov and Kosterin, 2003). The North American admirals and the Poplar admiral, in contrast, feed only on host plants from the Salicaceae (Platt, 1983; Scott, 1986). Therefore, given the monophyly of the Nearctic species, it is likely that the North American radiation of these butterflies is the result of a single migration event related to the expanding distribution of a novel host. Several lines of evidence support this view. First, aspen (the host plant of the Poplar admiral) is extremely widespread across all of Eurasia and North America and shows little evidence for differentiation between the two continents (Mitton and Grant, 1996). Second, the ancestral host plant of the Palearctic admirals, *Lonicera*, is Asiatic in origin and disperses poorly relative to many species of the family Salicaceae (Dubatolov and Kosterin, 2000; Grashof-Bokdam et al., 1998). Third, the Poplar admiral, which shares the wide geographic distribution of its larval host, has both adult wing pattern features and larval
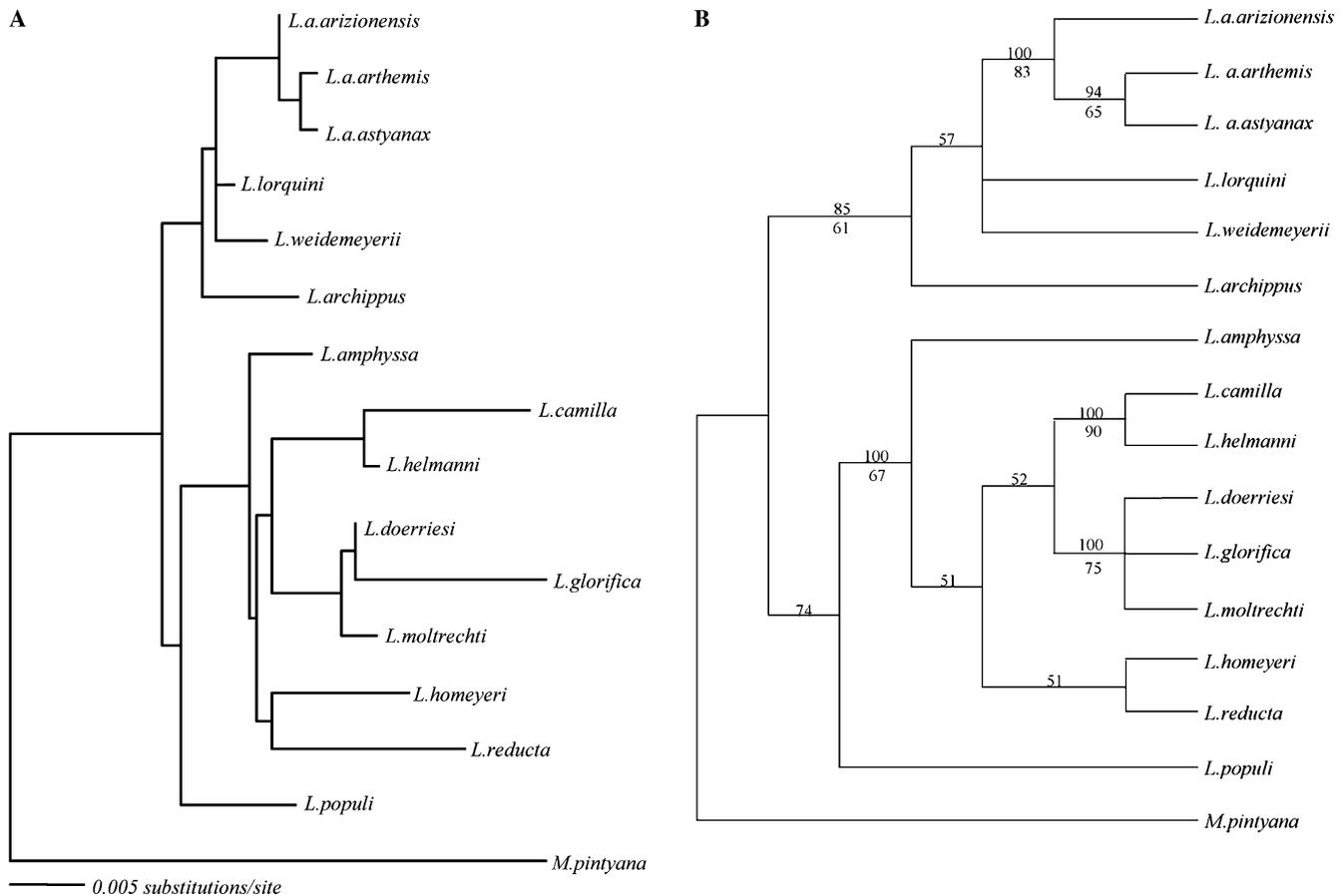
A

B

Fig. 3. (A) Maximum-likelihood phylogram of EF1-α sequences for sampled representatives of Nearctic and Palearctic admiral species (*Limenitis*); tree is rooted using *Moduza pintyana* as outgroup. (B) Bayesian consensus tree for EF1-α showing relationships between the Nearctic and Palearctic species of *Limenitis*. Numbers above the branches represent posterior probabilities for a given clade, those below are parsimony bootstrap values.

morphological characteristics (e.g., resemblance to bird droppings) that are similar to North American *Limenitis* (Willmott, 2003). If the host-switch to Salicaceae occurred in the common ancestor of the Poplar and North American admirals, then it provides a clear explanation of the spread of this lineage to the Nearctic. Initial colonization of the Nearctic by *Limenitis* may have been driven by range expansion of a novel host plant (Salicaceae) across the Bering land bridge to North America. Alternately, ancestral *Limenitis* may have made use of the pre-existing widespread distribution of the genus *Populus* (Hamzeh and Dayanadan, 2004) and then eventually colonized the Nearctic by dispersal and range expansion. Subsequent speciation and wing pattern evolution among Nearctic admirals clearly followed this colonization event and may have been facilitated by glacially enforced population subdivision and co-occurrence with chemically defended model species.

4.3. Relationships among North American taxa and the origins of mimicry

The recovered phylogenies indicate that a white-banded ancestor became established in North America and subse-quently resulted in several major lineages, three of which independently evolved mimicry (Figs. 2–4A). The mimetic Viceroy butterfly (*L. archippus*) is the basal lineage within North America, and is sister to two clades that each contain mimetic and non-mimetic wing pattern forms (Fig. 4A). While historically viewed as a Batesian mimic (Brower, 1958), later work has demonstrated that the Viceroy varies considerably in palatability over its range (Ritland, 1998; Ritland and Brower, 1991), and this species is now considered to be a Mullerian co-mimic of the Monarch butterfly (*Danaus plexippus*). Furthermore, theory suggests that it may be easier for a novel mimic to evolve phenotypic similarity to an aposematically colored model than to acquire chemical defensive via detoxifying and/or sequestering host plant compounds (Turner, 1981). Evolutionary changes in wing pattern, therefore, are expected to precede the origins of chemical defense. The basal position of the Viceroy is consistent with this hypothesis, assuming divergence was related to the origins of the mimetic phenotype, and suggests that its unpalatability may be more recently derived than its mimetic resemblance to the Monarch. This species has specialized within the broad plant family Salicaceae to feed exclusively on willow (*Salix* spp). Willow is known to contain numerous secondary chemical compounds that the
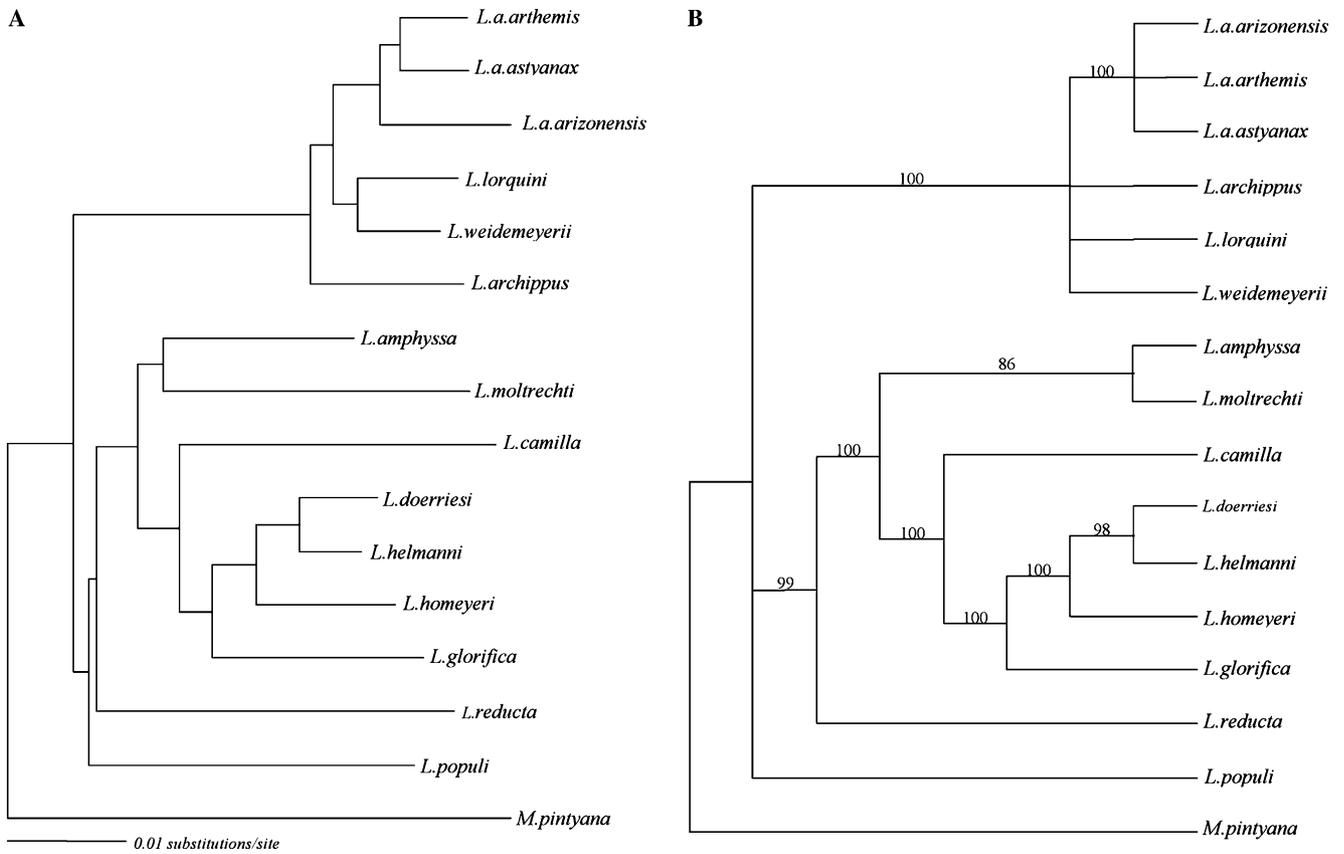
Fig. 4. (A) Maximum-likelihood phylogram based on the combined data from COI/II and EF1-α. (B) Bayesian consensus tree based on combined data from COI/II and EF1-α. Gene-specific and site-specific DNA substitution models produced identical topologies and only the by-gene consensus tree is shown here.

Table 3
Pairwise sequence divergence for mitochondrial COI/II (below gray boxes) and elongation factor 1-α (above gray boxes)

	Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	A. selenophora		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	L. a. arizonensis	10.0		0.2	0.2	1.5	1.0	2.6	1.9	2.6	2.0	2.2	0.4	2.0	1.4	—	2.3	—	0.7	4.0	—
3	L. a. arthemis	9.9	2.6		0.2	1.9	1.1	3.0	2.2	2.9	2.3	2.5	0.8	2.3	1.8	—	2.7	—	1.0	4.3	—
4	L. a. astyanax	10.0	1.8	2.6		1.9	1.1	3.0	2.2	2.9	2.3	2.5	0.8	2.3	1.8	—	2.7	—	1.0	4.3	—
5	L. amphyssa	8.3	7.9	7.3	7.9		1.8	2.2	1.1	2.1	1.2	1.3	1.3	1.1	1.5	—	1.8	—	1.5	4.5	—
6	L. archippus	9.4	3.6	3.9	3.4	7.3		2.7	2.1	3.0	2.2	2.4	0.8	2.2	1.7	—	2.5	—	1.0	4.2	—
7	L. camilla	10.0	8.5	9.0	8.8	6.3	9.2		2.0	3.0	1.1	2.4	2.4	2.2	2.2	—	2.8	—	2.6	5.3	—
8	L. doerriesi	9.1	8.3	8.1	8.2	5.3	7.8	5.5		1.2	1.1	1.4	1.7	3.0	1.9	—	2.0	—	1.9	4.5	—
9	L. glorifica	9.3	8.4	8.7	8.5	5.7	8.5	5.8	3.9		2.1	2.4	2.5	1.5	2.8	—	3.0	—	2.8	5.2	—
10	L. helmanni	8.9	8.1	8.0	8.0	5.0	7.6	5.1	1.2	3.2		1.8	1.8	1.3	2.0	—	2.1	—	2.0	4.6	—
11	L. homeyerii	8.9	8.2	8.1	8.0	5.1	7.7	5.3	2.6	4.3	2.2		2.0	1.3	2.0	—	2.0	—	2.2	4.5	—
12	L. lorquini	9.3	2.6	3.1	2.4	7.4	3.4	8.5	7.9	8.1	7.6	7.7		1.8	1.2	—	2.1	—	0.4	4.2	—
13	L. moltrechti	9.4	8.9	9.1	8.7	5.7	8.8	7.0	6.5	7.3	6.4	6.6	8.8		2.0	—	2.1	—	2.0	4.4	—
14	L. populi	9.5	8.3	8.5	8.3	6.8	8.2	7.7	6.2	7.2	6.0	6.6	8.1	8.1		—	1.9	—	1.4	4.1	—
15	L. pratti	8.5	11.4	11.8	11.5	10.0	11.2	10.7	10.5	11.8	10.4	10.8	11.3	11.2	10.9		—	—	—	—	—
16	L. reducta	9.2	8.6	8.9	8.8	6.6	8.6	8.2	7.3	7.6	7.0	7.1	8.2	7.8	10.9	10.6		—	2.3	5.3	—
17	L. staudingeri	9.8	8.8	8.4	8.9	8.5	9.2	9.2	9.3	9.8	8.9	9.0	9.1	10.0	9.1	11.3	9.3		—	—	—
18	L. weidemeyerii	8.9	2.5	3.3	2.6	7.2	3.1	8.4	7.8	8.3	7.6	7.4	2.0	8.4	7.7	11.0	8.2	8.5		4.4	—
19	M. pintyana	9.5	10.2	9.7	10.1	8.2	10.1	9.1	9.2	9.3	8.8	8.9	10.2	9.9	9.6	11.5	9.3	8.2	9.6		—
20	N. speyeria	12.3	12.6	12.5	12.4	10.4	12.2	11.1	11.0	11.8	10.5	10.9	12.5	11.8	11.0	11.2	11.6	12.4	11.9	11.5	

Viceroy larvae may sequester for chemical defense. Therefore, it is possible that this additional specialization in larval host plant diet was associated with the origins of chemical defense in this butterfly species.

The relationship between the two western wing pattern races of admiral butterflies in the United Sates, the mimetic Lorquin's admiral (*L. lorquini*) and the non-mimetic Weidemeyer's admiral (*L. weidemeyerii*), is not well

resolved (Figs. 2–4). However, neighbor-joining trees (not shown) and the combined maximum-likelihood tree suggest that the Lorquin's and Weidemeyer's admirals are sister taxa and shared a most common recent ancestor with the widespread and geographically variable eastern species complex *Limenitis arthemis*. The ambiguity regarding the relationship between these two western butterfly species is not new, however. Previous work utilizing allozymes showed extensive allele sharing between these wing pattern morphs, and Porter (1990) has argued that they be considered as geographic morphs of the same species. However, more recent work (Boyd et al., 2000) found a deficit of hybrid females between the two forms (*L. lorquini* and *L. weidemeyerii*) across one hybrid zone transect, suggesting that at least partial barriers to gene flow exist where these two wing pattern forms co-occur. In either case, the fact that they are able to maintain the distinctiveness of the their respective wing patterns in spite of occasional hybridization suggests the action of natural selection against intermediate phenotypes. If so, then the origin of mimicry within this lineage may be seen as contributing to current barriers to gene exchange at least for regions of the genome controlling wing pattern.

Historically, defining evolutionary relationships among Nearctic species of *Limenitis* has been controversial (Platt, 1983). Widespread reports of hybridization among nominal forms and a lack of rigorous molecular investigations of species boundaries in this group have limited the utility of these butterflies as a model system for wing pattern evolution and speciation. The debate over species boundaries has been most pronounced with respect to the *L. arthemis* complex. This polytypic species consists of two parapatrically distributed wing pattern morphs, the non-mimetic White-banded Admiral (*L. arthemis arthemis*) and the mimetic red-spotted purple (*L. a. astyanax*) and a third, allopatric mimetic population of red-spotted purples (*L. a. arizonensis*) in the southwestern United States. Early authors (Scudder, 1872; Edwards, 1861; Remington, 1958, 1968) considered the two eastern races of these butterflies to be closely related incipient species while later workers (e.g. Platt and Brower, 1968; Platt and Allen, 2001) have argued that they are conspecific because there is no evidence of postzygotic barriers and because Hardy–Weinberg analyses based on knowledge of the genetics of wing pattern indicates no heterozygote deficits.

While this study was not intended as an investigation of species boundaries within the *Limenitis arthemis* complex, it is worth noting that each of the three described wing pattern subspecies possesses unique and deeply divergent mitochondrial haplotypes. This suggests that these wing pattern populations are not simply geographical forms of a larger, panmictic species and that significant population structure exists among the three subspecies. Evidence for hybridization between *arthemis* and *astyanax* is restricted to one individual sampled near the contact between these two wing pattern races (a red-spotted purple phenotype from Virginia that possesses a mtDNA haplotype characteristic

of "white-banded" individuals). Presumably, this reflects mitochondrial introgression, known to occur in many pairs of hybridizing taxa (Harrison and Bogdanowicz, 1997; Dowling and Secor, 1997 for review). The apparent stability of the hybrid zone between these two morphs is consistent with the view that selection related to wing pattern mimicry may be isolating these taxa for at least some portion of their genomes.

The mitochondrial gene genealogy fails to strongly resolve the relationship among the three subspecies of the *Limenitis arthemis* complex but both this locus and data from EF1-α suggest that the allopatric, southwestern population (*L.a. arizonensis*) may have diverged first and that the two eastern morphs represent a separate and polymorphic lineage. The range and host breadth of the putative model for the red-spotted purple (*L. a. arizonensis* + *L. a. astyanax*) phenotype, the Pipevine swallowtail (*Battus philenor*), supports this view. The Pipevine swallowtail occurs throughout the entire southern United States and well south into Mexico (Scott, 1986). While this species feeds primarily on *Aristolochia serpentaria* and *A. reticulata*, the available host plant breadth in the southwestern United States is much larger than in the eastern US (Fordyce and Nice, 2003). Therefore, it is likely that the southeast, with is higher host plant diversity, served as the ancestral refuge of the model and that mimicry may have first evolved in this region of the United States. If so, then either mimicry within this complex evolved twice (once in the southwest and again in the southeast) or it was lost in northern White-banded populations of *L. a. arthemis*. Recent work (Mullen and Harrison, in preparation) suggests that multiple origins of mimicry may best explain the history of this complex and that selection related to mimicry limits gene flow between mimetic and non-mimetic populations.

4.4. Taxonomic status of the Nearctic and Palearctic Limenitis

Several authors (e.g., Scudder, 1872) have placed the Nearctic and Palearctic admirals in separate genera: *Basilarchia* for the North American forms and *Limenitis* for the Asian species. The molecular phylogenies reported here clearly support the distinctiveness of the Nearctic forms, but recognizing these species as a distinct genus may create a paraphyletic genus *Limenitis*, given the indeterminate position of *L. populi*. A possible solution is to treat the North American forms plus *L. populi* as the genus *Basilarchia* and restrict *Limenitis* to the remaining Palearctic species. Taxonomically, this arrangement is justified given the shared host plant use and the similarity in larval morphologies and adult wing patterns between the Nearctic species and *L. populi*. Furthermore, this taxonomic treatment would highlight the common evolutionary history of this group relative to other Eurasian *Limenitis* and provide a clear hypothesis of relationships for future work. In addition, it is clear even from the non-exhaustive sampling in this study that *Limenitis* as currently defined is polyphyletic

(Fig. 2A). *Limenitis pratti* has been recognized for its unique status and been moved (Gorbunov and Kosterin, 2003) to a new monotypic genus, *Seokia*, so the position of this species is unsurprising. *Limenitis staudingeri*, in contrast, is an endemic species restricted to several islands in the Indo-Pacific and has been traditionally seen as belonging to *Limenitis* sensu stricto. Based on its phylogenetic position in the mitochondrial genealogy and the fact that it has wing patterns typical of another genus in the subtribe Limenitidini, *Moduza*, it is clearly misplaced. Given the widespread confusion regarding phylogenetic relationships among species and genera within the subtribe Limenitidini, it is clear that there is a need for further revisionary taxonomic work of the Palearctic members of the genus *Limenitis* and other closely related groups.

4.5. Mimicry and speciation

The strongly supported monophyly of the Nearctic admirals and the relationships among mimetic and non-mimetic species of these butterflies within North America suggests that mimicry has been strongly associated with, or potentially a driver of, speciation within this group. Recent empirical evidence from nature (Allender et al., 2003; Funk, 1998; Noor, 1995; Rundle and Nosil, 2005; Schluter, 2001) suggests that speciation may often be a pleiotropic consequence of divergent natural selection in heterogeneous environments. Mimicry and warning coloration are classic examples of ecologically adaptive phenotypic traits thought to contribute to speciation in this way (Bates, 1862; Jiggins et al., 2001; Mallet and Joron, 1999; Mallet, 1993; McMillan et al., 1997; Naisbit et al., 2001, 2003). For instance, early work on Müllerian mimicry in neotropical *Heliconius* butterflies revealed that strong natural selection can maintain hybrid zones and reduce gene flow between mimetic wing pattern races (Mallet and Barton, 1989). In addition, recent work in this system has shown that strong premating barriers exist between both intra- and interspecific wing pattern races due to assortative mate choice based on mimetic wing patterns (Jiggins et al., 2001, 2004). Therefore, it is reasonable to hypothesize that natural selection on mimetic wing patterns among Nearctic *Limenitis* may have contributed to speciation in this group. Furthermore, current selection related to mimicry may also limit gene flow between mimetic and non-mimetic populations of these butterflies for at least some regions of their genomes.

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