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Conservation Genetics and the Implication for Recovery of the Endangered Mitchell's Satyr Butterfly, *Neonympha mitchellii mitchellii*

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Abstract

The modern delineation of taxonomic groups is often aided by analyses of molecular data, which can also help inform conservation biology. Two subspecies of the butterfly *Neonympha mitchellii* are classified as federally endangered in the United States: *Neonympha mitchellii mitchellii*, the Mitchell's satyr, and *Neonympha mitchellii francisi*, the Saint Francis's satyr. The recent discovery of additional disjunct populations of *N. mitchellii* in the southeastern US could have important implications for both legal protection and management decisions. We elucidated the relationships among 48 individuals representing 5 *N. mitchellii* populations using 6 molecular markers (5 nuclear and 1 mitochondrial) under a variety of analytical frameworks. Phylogenetic analysis resulted in moderately supported clades that corresponded with the geographic region where samples originated. Clustering analyses identified 3 groups, wherein the 2 named subspecies formed separate clusters. Coalescent analyses indicated evolutionary divergence between *N. m. mitchellii* and all other populations but weakly supported divergence among *N. m. francisi* and the recently discovered populations. Hence, the 2 currently accepted subspecies were clearly different from one another, but the recently discovered populations could not be completely distinguished from *N. m. francisi* or each other. We propose that *N. m. mitchellii* and *N. m. francisi* continue to be managed as separate endangered species.

Key words: *conservation genetics, endangered species, lineage sorting,* Neonympha

Taxonomic groups have traditionally been delineated using observed morphological differences among taxa (Agapow et al. 2004). This paradigm has stood for hundreds of years and served as a foundation for systematics and as the first species concept (that similar looking individuals were part of the same species) (Kaesuk-Yoon 2010). Although species concepts are ideological constructs and may seem esoteric to some (i.e., the arbitrary choice of characters used to separate taxa), they do have real world consequences. For example, in the first edition of Holland's 1898 *Butterfly Book*, there were 47 species listed in the genus *Speyeria* where there are 13 at present, and in Europe, every local population of the *Apollo* butterfly was given a unique name (Shapiro 2002). Indeed, taxonomic inflation has been especially pronounced within the Lepidoptera, where the trade value of an organism often depends on its rarity or uniqueness (Kristensen et al. 2007; Laufer 2010; Leach 2013). Taxonomic delineation is especially important for threatened and endangered species, particularly in the United States where legal protection tends to be focused on taxonomy rather community or habitat assemblages (Scott et al. 2006).

Although the choice of morphological characters used to distinguish a species may be arbitrary, a variety of empirical methods exist for the delimitation of species. Broadly, these methods can be considered "tree" and "nontree" based methods (Sites and Marshall 2004). Although they make use of different criteria, a unifying theme among many of these methods is that a lineage is the unit we seek to identify (Wiens 2007). Often, some methods yield conflicting results

when applied to the same data, but by using multiple methods, researchers can provide a level of cross-validation and corroborative evidence for a hypothesis (Agapow et al. 2004).

When enacted, the Endangered Species Act (ESA) of 1973 minimally defined the term "species" to the point where some legal scholars have called the ESA's definition "singularly uninformative" and "merely a list masquerading as a definition" (Doremus 1997). The only criterion given to federal agencies was that the "best available science" be employed to determine whether a species warranted protection (Doremus 1997). Litigation centered on the ESA has tended to focus on whether or not the federal agencies have met their statutory obligations in the decision-making process and not whether the taxon in question is a good species (Shapiro 2002).

Recognizing that genetic variability is especially important to endangered species, molecular genetics been increasingly employed to aid in the conservation and delimitation of taxa (Avise 1989). Indeed, morphological and molecular differences are often highly correlated (Lefébure et al. 2006). Molecular data have been used for some time to identify genetically distinct populations and, more recently, to estimate historic gene flow and the timing of vicariance events (Hedrick and Miller 1992; Forister et al. 2011). Molecular approaches, when combined with studies of natural history and ecology, have been particularly powerful and helped to resolve otherwise intractable issues (Forister et al. 2011).

The Mitchell's satyr, *Neonympha mitchellii mitchellii* French 1889, and the Saint Francis's satyr, *Neonympha mitchellii francisi* Parshall and Kral 1989, are 2 endangered species of butterfly found in the eastern United States (Hamm et al. 2013). The Mitchell's and Saint Francis's satyrs were placed into different subspecies based on ecological differences such as habitat and voltinism and phenotypic differences such as wing size and the number of hindwing border ocelli (Parshall and Kral 1989). Cladistic analyses of mitochondrial cytochrome oxidase I (COI) and II sequences using a small sample of individuals from throughout the range yielded inconclusive results as to the relationship of the populations (Goldstein et al. 2004). More recently, data from a geometric morphometric analysis indicated that *N. m. mitchellii* and *N. m. francisi* could be distinguished based on differences in wing pattern (Hamm 2012), lending quantitative support to the distinction. Under the ESA, endangered invertebrates (whether named species or subspecies) are treated as full species wherever they are found; thus for the remainder of this work, we will use the term "species" when referring to *N. m. mitchellii* and *N. m. francisi* even though they are taxonomically subspecies. As of 2013, the Mitchell's satyr was found at 18 isolated sites across lower Michigan and northern Indiana, though it historically also occurred in Ohio, New Jersey, Wisconsin, and possibly Maryland (Figure 1) (Hamm et al. 2013). The Saint Francis's satyr has only been known from 1 site (260 km^2) in North Carolina (Figure 1) (Kuefler et al. 2008). Each endangered species has a recovery plan that outlines the minimum number of "viable populations" required before the species are no longer protected (USFWS 1996, 1998). If additional populations of either species were to be discovered, it would likely affect their protected status.

In 1998, a butterfly, identified as *N. mitchellii*, was observed in southwestern Virginia, and subsequent field surveys identified 17 distinct sites where the butterfly was present (Roble et al. 2001). These sites are ~200 km from the *N. m. francisi* site in North Carolina, and they are separated by watersheds and geophysical features that would likely precluded recent migration given this species propensity for short-range dispersal (<1 km) (Figure 1) (Hamm et al. 2013). During the summer of 2000, additional *N. mitchellii* butterflies were discovered in central Alabama (Glassberg, 2000), which prompted the US Fish and Wildlife Service to commission a survey that ultimately identified 15 additional sites in Alabama and 3 in northeastern Mississippi (Hart 2004). Individuals from these recently discovered populations could not be included in the geometric morphometric analysis because no museum specimens exist and their protected status precluded the possibility of obtaining samples. None of the recently discovered *N. mitchellii* populations have been formally assigned to a taxonomic group below the species level, but all have had federal protection extended to them until their rank can be firmly established. If any of the recently discovered *N. mitchellii* populations were assigned to either of the protected species, the recovery criteria (of 25 viable populations for *N. m. mitchellii*, USFWS 1998; and 3 additional metapopulations for *N. m. francisi*, USFWS 1996) for each species could be considered fulfilled and federal protection under the ESA removed.

In this work, we sought to assess the taxonomic status of both previously recognized subspecies and all recently discovered populations based on the analysis of genetic variation from 1 mitochondrial and 5 nuclear loci. We specifically addressed 2 questions: 1) is there geographically organized genetic differentiation; and 2) do the endangered taxa form evolutionarily distinct lineages from other *N. mitchellii*? Although we would prefer to adhere to the biological species concept (a definition based on a process), as diagnosed by fitness of hybrids in natural populations (Mayr 1942), we could not conduct the necessary experimental crosses due to the protected status of this group. As such we relied on observed patterns of variation to distinguish species.

Materials and Methods

Collection and Preparation of Samples

Genetic samples were nonlethally collected from *N. mitchellii* populations in the Eastern United States (Hamm et al. 2010). A total of 48 samples, collected during 2008 and 2009, were analyzed for this study: Alabama (AL) (n = 5), Indiana (IN) (n = 2), Michigan (MI) (n = 25), Mississippi (MS) (n = 3), North Carolina (NC) (n = 7), and Virginia (VA) (n = 6) (Figure 1). All samples were collected according to conditions specified in USFWS permit TE-175852 and other agency permits. The genetic samples, which consisted of a 2-mm piece of the A3 vein from the hind wing, were placed directly into 95% EtOH using sterilized forceps, and DNA was extracted within 48 h using a DNEasy® Blood & Tissue Kit (Qiagen Inc., Valencia, CA) or a ZR Tissue & Insect

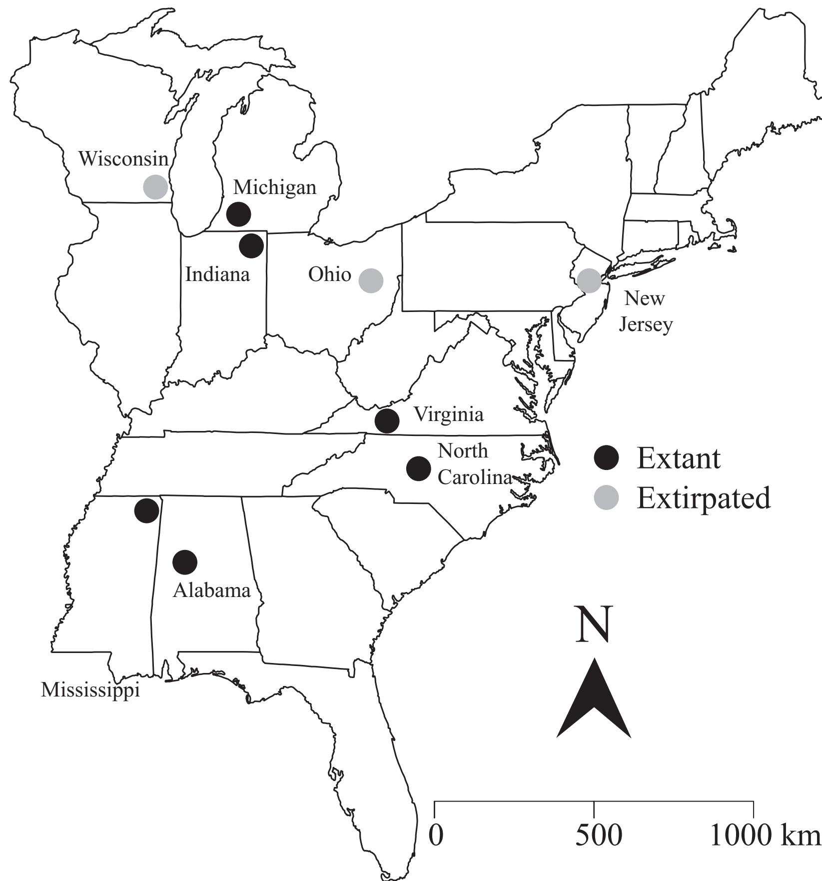

Figure 1. Map of known *Neonympha mitchellii* populations in the United States and sample sizes for this study. Black dots indicate areas with extant populations (Michigan [25], Indiana [2], North Carolina [7], Alabama [5], Mississippi [3], and Virginia [6]); grey dots indicate areas with extirpated populations (Ohio and New Jersey).

DNA Kit™ (Zymo Research Corporation, Irvine, CA) and then stored at −80 °C. Outgroup sequence data were gathered from samples of *Neonympha areolata*, the putative sister taxon to *N. mitchellii*, which were collected from Alabama as part of this study, as well as *Taydebis* sequences that were acquired from museum specimens (collected by P. J. DeVries) or Genbank.

PCR Amplification DNA Sequencing

We sequenced 6 loci for all 48 *N. mitchellii* individuals and the outgroup taxa, *N. areolata* and *Taydebis*, which was identified as a closely related ancestral taxon to *Neonympha* (Wahlberg et al. 2009): the mitochondrial gene COI (a longer section of this marker than Goldstein et al. 2004); the nuclear genes elongation factor 1α (EF-1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein S5 (RpS5); and 2 anonymous single copy nuclear loci AL15_16, and AL20_21 (Hamm 2011)(Table 1). PCR products were visualized by gel electrophoresis and prepared for direct sequencing with ExoSAP-IT® (USB Products, Santa Clara, CA) and directly sequenced on both strands using an ABI Prism 3730 (Life Technologies Corporation, Carlsbad, CA) at the Research Technology Support Facility at Michigan State University. All anonymous loci were cloned into pGEM vector (Promega Corp., Madison, WI), were transformed into *E. coli* DH5α, and were then sequenced forward and reverse using M13 primers. Resulting chromatograms were assembled into contigs for each individual separately in the program Geneious v5.6 (Drummond et al. 2012) and visually inspected, and polymorphisms were called only if both reads were in agreement. Sequences were used as queries for BLASTn searches (Altschul et al. 1990) within the "nr" database at NCBI to confirm orthology. Consensus sequences from each contig were then aligned using the program Clustal W (Thompson et al. 1994), and the assemblies were visually inspected for evidence of linkage and recombination.

Phylogenetics

To establish monophyly for *N. mitchellii*, we utilized 2 phylogenetic approaches, maximum likelihood and Bayesian concordance, to distinguish major phylogenetic groups from one another, designating *Taydebis* as the outgroup. All loci were examined for neutrality against $\alpha = 0.05$ using Tajima's D in the program DnaSP 5.1 (Librado and Rozas 2009). We used the program MrModeltest 2.3 (Nylander 2004) to select models of molecular evolution for each locus

Table 1 Descriptive information and statistics per locus used to generate sequence data for all *Neonympha* butterflies for this study

Locus	Primers (forward/reverse)	Length (bp)	# Haplotypes	S	π^h	θ^h	AIC best model[a]	Accession number
COI	CI-J-2183/Pat2[b] LCO 1490/HCO 2198[c]	689	9	15	3.35×10^{-3}	1.56	HKY + I	KC112124–KC112171
EF-1α	ef44/ef51r[d] ef51.9/efrcM4V[d]	590	11	9	1.94×10^{-3}	1.36	SYM	KC112076–KC112123
GAPDH	GAPDH_F1/ GAPDH_R2[e]	635	6	9	2.74×10^{-3}	0.78	SYM	KC112220–KC112267
RpS5	RpS5_F1/RpS5_R1[e]	249	2	1	0.16×10^{-3}	0.15	K80	KC112172–KC112219
AL15_16	2-H9-F1/2-H9-R1[f]	576	10	19	8.53×10^{-3}	3.31	F81 + I	KC112268–KC112315
AL20_21	2-E12-F1/2-E12-R1[g]	272	2	1	0.15×10^{-3}	0.19	F81	KC112316–KC112363

S is the number of variable sites, π is the nucleotide diversity, and Watterson's θ per sequence.

[a] AIC model comparisons conducted in the program MrModeltest 2.3 (Nylander 2004). Primer sequences provided in supplemental material.

[b] Simon et al. 1994.

[c] Folmer et al. 1994.

[d] Monteiro and Pierce 2001.

[e] Based on Wahlberg and Wheat 2008 though modified for this study.

[f] Hamm 2011.

[g] Developed for this study, using methods described in Hamm 2011.

[h] Parameters estimated in the program DnaSP (Librado and Rozas 2009).

(partition) within the data set, and optimal models were selected based on Akaike's Information Criterion (Posada and Buckley 2004) (Table 1). We used the program RAxML v7.2 (Stamatakis 2006) to construct a partitioned maximum likelihood tree, with each locus allowed to evolve under a unique model of molecular evolution. The most likely tree was determined based on 2000 independent starting trees, and branch support was assessed based on 10 000 bootstrap pseudoreplicates.

We examined the concordance of gene trees under a Bayesian framework. First, we explored the tree space of each locus using the program MrBayes 3.2 (Ronquist and Huelsenbeck 2003) using 2 Markov Chain Monte Carlo (MCMC) simulations with 4 chains of 2M generations each, the results of which were analyzed after the first 25% of trees were removed as burn-in. We considered the runs to have converged once the standard deviation of the split frequencies was below 0.01 and the 2 runs produced identical topologies. Concordance among the resulting gene trees was determined using the program BUCKy v1.4 (Larget et al. 2010) with 1 million MCMC simulations and 4 chains across a range of α priors (0.1, 1, and 100).

Population Assignment

To prepare the data for population-level analysis, we used the program PHASE 2.1.2 (Stephens et al. 2001) to estimate haplotypes for genotypes that were heterozygous across multiple sites and invoked the –d1 argument to accommodate multiallelic data without the stepwise mutation model. We used the Bayesian algorithm implemented in the program STRUCTURE 2.3 (Hubisz et al. 2009) to assign individuals to clusters using data from the 4 loci that were variable within *N. mitchellii*. We tested clustering models that had complete admixture with both correlated and uncorrelated allele frequencies and a model with no admixture and uncorrelated allele frequencies, the latter of which we considered to be the most biologically plausible. We determined the optimal number of clusters (K) by conducting MCMC simulations for 100 000 iterations with a 10 000 iteration burn-in for $K = 1$ through $K = 10$ with simulations for each K value repeated 10 times. We determined the optimal K for each model using the ΔK method (Evanno et al. 2005), which uses the rate of change of log probability for successive values of K to estimate the optimal number of clusters. Although each MCMC simulation was run under the same parameters, some stochasticity, such as label switching, is expected (Stephens 2000). To account for label switching, the assignment probabilities from the ΔK best model were averaged over the 10 runs using the "FullSearch" option in the program CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007).

To investigate the population genetic history of *N. m. mitchellii*, we used the program IMa2 (Hey 2010) to estimate splitting times between regional populations under an isolation with migration model (Hey and Nielsen 2004). We conducted pairwise coalescent simulations between *N. m. mitchellii* and the clades and clusters identified by the previously described analyses. We ran each comparison using an exponential migration prior (-j7 option), 20 MCMC chains with geometric heating (heating parameters: $a = 0.96$, $b = 0.9$), and at least 20M steps where at least 100 000 genealogies were saved after a burn-in of 100 000. We confirmed a lack of pattern in the parameter estimates over time by visually inspecting trend plots from each run to ensure that sufficient chain mixing had occurred. Priors were adjusted after initial runs to ensure that the posterior distributions of parameter estimates were not sensitive to the priors. All data

sets and statistical code required to replicate these results are freely available on the Dryad Data Repository (doi:10.5061/dryad.n31kq).

Results

Genetic Diversity

We sequenced a total of 2933 base pairs from 6 loci for all 48 *N. mitchellii* individuals, 1 *N. areolata* and 1 *Taydebis* (Table 1). No insertions or deletions were observed in the data, and the sequences were easily aligned. Nucleotide BLAST results exhibited strong sequence identity (E value = 0.0) to putative butterfly orthologs. The Tajima's D test failed to reject neutrality for all loci ($P > 0.05$), and all sequences were included in subsequent analyses. Estimates for the number of haplotypes, number of variable sites, nucleotide diversity, Watterson's theta, and the results of AIC model selection are presented in Table 1. Polymorphism varied greatly by locus: for example, 2 of the loci (RpS5 and AL20_21) were invariant within *N. mitchellii*, whereas each of the remaining loci had at least 9 variable sites where nucleotide diversity ranged from 0.15×10^{-3} to 8.53×10^{-3}. For all loci, inferred haplotypes were consistent across replicated runs in PHASE.

Phylogenetics

Based on maximum likelihood analysis, we found that *N. mitchellii* formed a monophyletic group and that individuals were assigned to clades based on the geographic region from which they were sampled, though with varying levels of bootstrap support (Figure 2A). Analysis using Bayesian concordance resulted in identical phylogenetic relationships as were observed in the maximum likelihood tree, though with low levels of concordance among the gene trees (Figure 2A). Both phylogenetic methods support monophyly for *N. m. francisi* and *N. m. mitchellii*, the 2 endangered taxa. Monophyly was also supported for samples from the remaining 2 regional lineages (AL/MS, VA), though the order of the branching was not strongly supported (Figure 2A).

Population Assignment

The sequence data generated from the cloning of alleles for the anonymous loci were used as a positive control for program PHASE, and in all cases, program PHASE correctly estimated haplotypes. The program STRUCTURE produced similar patterns of clustering across all models and values of K. The optimal number of clusters was $K = 3$ (Figure 2B) for

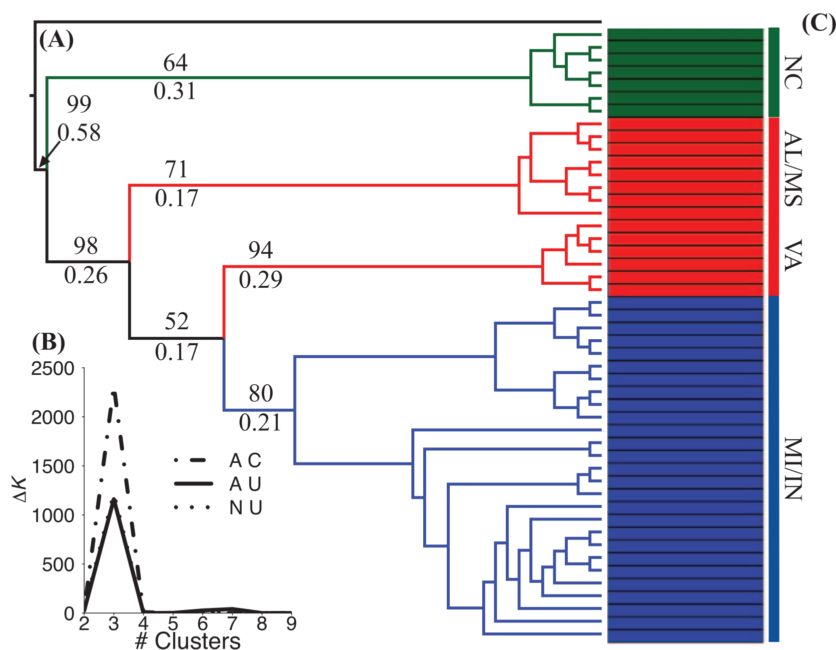

Figure 2. (**A**) Phylogenetic tree depicting the relationships among *Neonympha mitchellii* butterflies. States are North Carolina (NC), Alabama (AL), Mississippi (MS), Virginia (VA), Michigan (MI), and Indiana (IN). Numbers above the branches represent support values based on 10 000 bootstrap pseudoreplicates on the ML tree generated in the program RAxML, and numbers below the branches represent Bayesian concordance values generated by the program BUCKy. The colors of the branches correspond to the clusters described in (**C**). The first branch at the base of the tree represents the outgroup. (**B**) Plot of ΔK values for the 3 models of population structure examined: population admixture with correlated allele frequencies (AC), population admixture with uncorrelated allele frequencies (AU), and no population admixture with uncorrelated allele frequencies (NU). (C) Bar plot depicting assignment probability from STRUCTURE analysis using a model with no admixture and uncorrelated allele frequencies and $k = 3$ clusters, though all models tested generated the same clustering pattern. Each bar plot represents the proportion of that individual's genome derived from a certain cluster.

all models using the ΔK method of Evanno et al. (2005). All 3 models examined through STRUCTURE resulted in the same pattern of clustering with high assignment probabilities (Figure 2C). This consistent pattern was such that all individuals from North Carolina (*N. m. francisi*) clustered together, all individuals from Michigan and Indiana (*N. m. mitchellii*) clustered together, and individuals from Virginia, Alabama, and Mississippi clustered together (Figure 2C).

Coalescent analysis resulted in posterior probability distributions for the splitting time parameter in pairwise comparisons among population clusters, the peaks of which ranged from narrow to broad, which indicated varying levels of support for each comparison (Figure 3). The distribution of posterior probabilities for the *N. m. mitchellii* (Michigan and Indiana) and *N. m. francisi* (North Carolina) comparison did not overlap with zero (Figure 3A), consistent with a lack of gene flow between populations. Similarly, the divergence time posterior distribution from the comparison between *N. m. mitchellii* with those from Alabama and Mississippi also did not overlap with zero. Although the comparison between *N. m. mitchellii* and *N. mitchellii* from Virginia generated a splitting time posterior distribution that partially overlapped with zero (Figure 3A), the 94% confidence interval (CI) for the splitting time did not include zero (though the 95% CI did). Pairwise comparisons among the remaining populations resulted in splitting time posterior distributions that overlapped with zero but tended toward very flat distributions, most likely caused by low statistical power in our sample with respect to these pairwise comparisons.

Discussion

The recently discovered populations clearly fell within the species *N. mitchellii*; however, we were unable to fully resolve the evolutionary history among regional populations. Although *N. m. mitchellii* (Michigan and Indiana) was distinct from all other regional populations, the relationships among the remaining regional populations were not clear across all analytical methods. Results from concordance and coalescent analyses, variation in signal among loci, and low sequence variation suggested that divergence among populations was relatively recent, which likely resulted in incomplete lineage sorting among alleles. The molecular data confirm that all

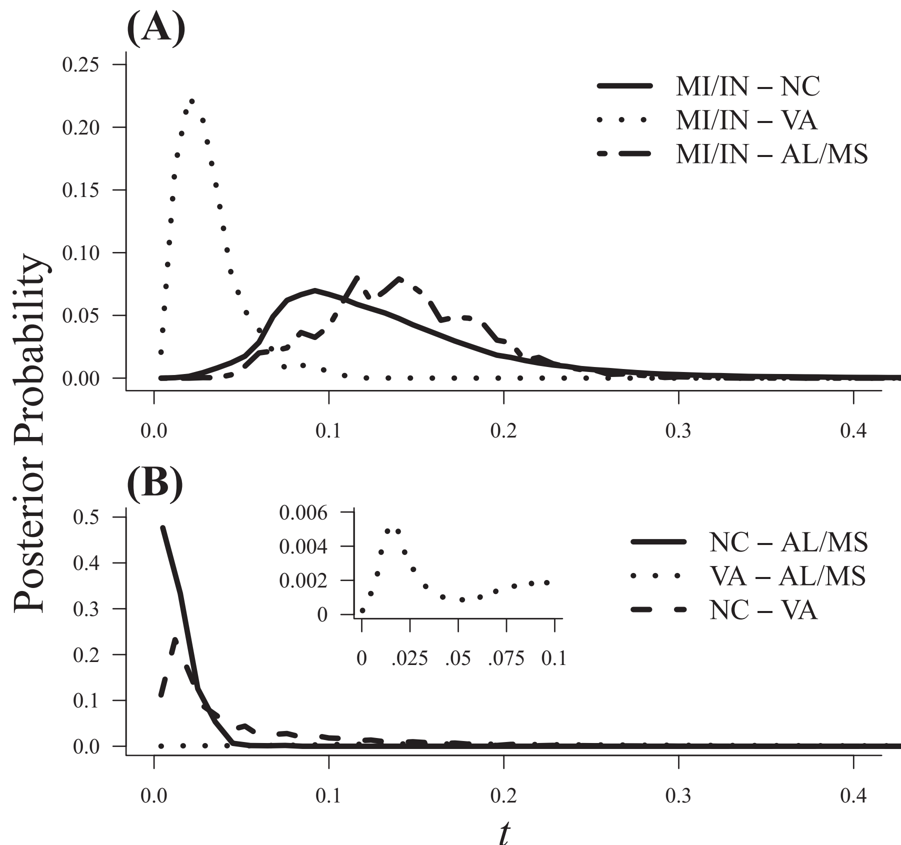

Figure 3. Posterior probability distributions of splitting times for (**A**) *Neonympha mitchellii mitchellii* (MI/IN) comparisons to *Neonympha mitchellii francisi* (NC), and the recently discovered populations in AL/MS and VA; (**B**) *N. m. francisi* to the recently discovered populations. Inset is the expanded VA–AL/MS comparison. States are North Carolina (NC), Alabama (AL), Mississippi (MS), Virginia (VA), Michigan (MI), and Indiana (IN). *t* Represents coalescent time units, which is dependent on the number of generations and effective population size (N_e).

recently discovered populations are members of *N. mitchellii*. Even the loci that were invariant within *N. mitchellii* had fixed differences relative to *N. areolata*, the putative sister taxon (Figure 2A). Because these recently discovered populations clearly fall within the presently recognized *N. mitchellii* clade, they should be included in all subsequent studies concerning the population structure and conservation status of the species as a whole.

The 2 endangered taxa, which were originally differentiated using ecological and morphological characters, were clearly distinguished using molecular characters, and we conclude that they are evolutionarily distinct. Phylogenetic, clustering, and coalescent methods all indicated that the 2 endangered species are different (Figures 2 and 3A), which is important because the initial distinction that lead to the taxonomic recognition of 2 different subspecies was made using largely qualitative data and a limited sample size (Parshall and Kral 1989). Our data provide a more compelling form of historical information in support of the taxonomic status for these subspecies. Indeed, the phylogenetic, concordance, and coalescent analyses indicated that these subspecies were among the most distantly related populations examined. It is important to note that the statistical support for clades was moderate, but that posterior probability for splitting time from coalescent analysis did not include zero (Figure 3).

In the case of the Virginia and North Carolina populations, geographic distance was not predictive of genetic distance (Figures 1, 2A, and 3). Though the 2 areas are only a few hundred kilometers apart, they are members of separate river drainages (Hamm et al. 2013). As these are wetland-associated butterflies that do not disperse far, this pattern suggests that ancestors of Virginia *N. mitchellii* radiated along the western side of the Appalachians following the Pleistocene, as has been proposed for other butterflies and skippers (Shapiro 1970, 1977).

When we interpreted our data in light of the newly discovered populations, the taxonomic distinctions among regional populations become more complex. One consistent result from the analyses was that the *N. m. mitchellii* clade was distinct from all other regional populations. The inconsistencies with respect to the remaining populations included the separation of *N. m. francisi* from Alabama/Mississippi populations based on coalescent analysis (Figure 3B), where divergence time distributions overlapped with zero (Figure 3). Finally, the phylogenetic and concordance analyses differentiated between the Alabama/Mississippi and Virginia regional populations (Figure 2A), whereas clustering and coalescent methods did not (Figures 2C and 3B). We further note that posterior probability of splitting time between Virginia *N. mitchellii* and *N. m. francisi* had distributions that did not overlap with zero but were extremely flat and indicated that our data lacked the power to address this comparison in a coalescent framework.

The 3 regional populations characterized by clustering methods (*N. m. mitchellii*, *N. m. francisi*, and Virginia plus Alabama/Mississippi) (Figure 2C) are distinguishable with our data, but historical demographic factors combined with recent reductions in the number and size of populations have likely resulted in the observed inconsistencies and/or low statistical support among methods of genetic analyses. Each of the methods has obvious differences in the type of historical signal that is detected, and these data appear to have been particularly exemplary in highlighting those differences. For example, statistical support from phylogenetic and concordance methods relies more heavily on the presence of shared derived characters and their prevalence within the data set (Felsenstein 2004). Because some of the loci had few informative characters (there were 48 variable sites within *N. mitchellii* among 2 093 characters, though 2 loci were invariant), branch support and concordance values were low, despite a consistent signal in the few informative characters present. The overall lack of informative sites resulted in many bootstrapped pseudoreplicate data sets without informative sites for subsets of branches, which resulted in low bootstrap support. Similarly, many of the gene trees sampled during the MCMC analyses were unresolved with respect to some of the ancestral branches, which resulted in low concordance values. Alternatively, had the data harbored sites with consistent support for alternative topologies, the concordance tree results would have exhibited moderate support for these alternative topologies (Rokas et al. 2003). Clustering analyses do not rely on synapomorphies but are more sensitive to multilocus genotypes and their frequencies in the sample (Falush et al. 2003). Clustering separated *N. m. mitchellii* and *N. m. francisi* from the recently discovered populations but classified the remaining populations as 1 cluster (Figure 2B).

The variance in signal across loci was also a likely contributor to the differences in results across methods. The lack of variation in 2 of the loci (GAPDH and AL20_21), which exhibited standard levels of divergence from the sister species, combined with the relatively low haplotype variation at the mitochondrial locus (COI), relative to other butterflies (de Jong et al. 2011), is consistent with a historically small effective population size. The informative sites present in COI exhibited the lowest levels of genetic diversity in *N. m. mitchellii*, despite the much larger sample size ($n = 25$; Table 1). Mitochondrial loci have a smaller effective population size than nuclear loci in animals due to uniparental inheritance and a lack of recombination. This reduced effective population size should, on average, result in greater reductions in genetic variation due to population bottlenecks compared with nuclear loci. Further complicating interpretation of the signal from COI is the fact that the bacterial endosymbiont *Wolbachia* is present in *N. mitchellii* at unknown levels (Hamm et al. 2013). We suspect that this is why the *N. m. mitchellii* exhibited the lowest levels of genetic variation at COI, and given the derived position of that regional population on the topology, may be indicative of small colonizing populations in the formerly glaciated states of Michigan and Indiana. The nuclear loci exhibited shared haplotypes among geographically disparate populations, which combined with the evidence of recent divergence among all clades lead us to infer that these populations are likely exhibiting incomplete lineage sorting among alleles. Although we were able to infer

some consistent patterns from these data, we also suspect that stronger statistical support for the postulated relationships and historical demography among all populations will require further study.

Based on our results, we believe that current conservation practices should continue such that *N. m. mitchellii* and *N. m. francisi* are managed as separate endangered species. Indeed, the one consistent result across all analytical methods is that *N. m. mitchellii* is different from all other *N. mitchellii*. Although the newly discovered populations in Alabama, Mississippi, and Virginia are clearly not *N. m. mitchellii*, their relationship to *N. m. francisi* is more ambiguous when examined using pairwise coalescent analyses; however, phylogenetic and clustering results suggested that the newly discovered populations are separate from *N. m. francisi*. Until we derive a more powerful data set, with the ability to resolve the relationships among regional populations, we refrain from making or recommending taxonomic changes for *N. mitchellii*, but we do recommend the *N. mitchellii* found in Virginia, Alabama, and Mississippi should not be managed as either of the endangered species.

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