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Speciation, ecological divergence, and phylogeny in plethodontid salamanders

A Dissertation Presented

by

Margaret Caitlin Fisher-Reid

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

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in

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Abstract of the Dissertation

Speciation, ecological divergence, and phylogeny in plethodontid salamanders

by

Margaret Caitlin Fisher-Reid

Doctor of Philosophy

in

Ecology and Evolution

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Speciation has been a subject of great interest and study since the founding of evolutionary biology by Darwin in 1859. In my thesis, I examine speciation from multiple perspectives in plethodontid salamanders.

Molecular phylogenies based on combined mitochondrial and nuclear DNA are commonly used to infer patterns of speciation. Yet, we know little about how mitochondrial and nuclear DNA interact to produce a combined-data tree. How much do trees from mitochondrial and nuclear DNA differ? How are topological conflicts between these data types typically resolved in the combined-data tree? I compare mitochondrial, nuclear, and combined-data trees for 14 vertebrate clades (including new nuclear data and analyses for the salamander genus *Plethodon*) in order to address these and other related questions. I find that while there is a large amount of conflict between mitochondrial and nuclear trees, these conflicts are often weakly supported and are often resolved in favor of nuclear data (despite typically having fewer variable characters), with the important exception of *Plethodon*.

The climatic niche is an important trait which has been implicated in speciation in a wide variety of organisms. However, the relationship between rate of climatic-niche evolution and climatic-niche breadth has not been explicitly tested. Using a 250 species phylogeny of Plethodontidae, and accompanying climatic niche data, I test this relationship. Generally, I find no relationship between rate of climatic-niche evolution and climatic-niche breadth. However, I

did find a strong, positive relationship between rate and breadth for single climatic variables (e.g., annual precipitation).

Finally, it is widely accepted that species can arise in allopatry and then later become sympatrically or parapatrically distributed. Patterns in the opposite direction are also possible, but have rarely been shown. In a multi-faceted analysis of *Plethodon cinereus* on Long Island, I show that two generally sympatric color morphs appear to have become parapatrically distributed. Additionally, the pure-lead populations on Long Island are divergent from other populations, suggesting incipient speciation. The distribution seems to be related to the different ecological preferences of the two morphs. These results suggest that spatial segregation of sympatric ecotypes might play an important part in parapatric speciation.

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List of Abbreviations

Chapter 1:

mtDNA……………………………………………………..……mitochondrial DNA
nucDNA………………………………………………………..….nuclear DNA
Pp……………………………………………………………...posterior probability

Chapter 2:

PCA…..……………………………………………...…principal component analysis
BMN……………subgenera *Bolitoglossa, Mayamandra, Nanotriton* (genus *Bolitoglossa*)
Ch………………………………………………………………genus *Chiropterotriton*
El………………………………………………subgenus *Eladinea* (genus *Bolitoglossa*)
ILPP…………………...…genera *Ixalotriton, Lineatriton, Parvimolge, Pseudoeurycea*
MOP…………... subgenera *Magnadigitata, Oaxakia, Pachymandra* (genus *Bolitoglossa*)
No…………………………………………………………………genus *Nototriton*
Oe……………………………………………………………..………genus *Oedipina*
An……………………………………………………………………genus *Aneides*
Ba……………………………………………...……………genus *Batrachoseps*
De……………………………………………………….…………genus *Desmognathus*
Eu………………………………………………...………………genus *Eurycea*
GPS…………………………...………genera *Gyrinophilus, Pseudotriton, Stereochilus*
Pc……………………………………………………..*Plethodon cinereus* species group
Pg……………………………………...…………*Plethodon glutinosus* species group
Pww………………………...………………*Plethodon welleri-wehrlei* species group
wP………………………………...………………western *Plethodon* species group

Chapter 3:

LI…………………………………………………...…………………Long Island
PCA…..……………………………………………...…principal component analysis
CG…………………………………………………...…………………costal groove
SVL……….……………………………………………………...…snout-vent length
TL..…………………………………………………...…………………tail length
AG……………………………………………...…………………axilla-groin length
SG..……………………………………………...…………………snout-gular length
HW…………………………………………………………...…………head width
TW…………...…………………………………...……………………trunk width
HLL…………………………………………………….........………hindlimb length
FLL…………………………………………………...……………forelimb length

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I hereby state that permission has been granted by myself and Dr. John J. Wiens (dissertation advisor) to reproduce the article "What are the consequences of combining nuclear and mitochondrial data for phylogenetic analysis? Lessons from *Plethodon* salamanders and 13 other vertebrate clades," published in the open-access journal *BMC Evolutionary Biology* in October 2011.

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I hereby state that permission has been granted by myself, Dr. Kenneth H. Kozak, and Dr. John J. Wiens (dissertation advisor) to reproduce the article "How is the rate of climatic-niche evolution related to climatic-niche breadth?" which is *in press* in the journal *Evolution* as of June 2012.

Introduction

Evolutionary biology and ecology are two deeply intertwined subjects that often benefit from joint study. Speciation, the origination of new species, is formally rooted in evolutionary biology, but its ties to ecology go back to Charles Darwin. Darwin recognized that natural selection was not only involved in change within species, but in their splitting as well (Coyne and Orr 2004). Both speciation and ecology benefit from being studied in a phylogenetic framework, which has been made much easier in our age of fast computing and relatively cheap DNA sequencing. In this dissertation, phylogeny, ecological divergence between species, and speciation (and the intersections of these subjects) are examined from several perspectives in plethodontid salamanders.

Speciation research is heavily based in phylogenetic methods. Indeed, the only figure in Darwin's (1859) *Origin of Species* is a phylogeny, and it symbolizes one of the two major premises of the *Origin*: common ancestry of all organisms (the other being natural selection). With phylogenetic methods, we can reconstruct a partial history of speciation that allows us to ask further questions about the ecological and evolutionary processes that play a role in the origin of species. Molecular phylogenies based on combined mitochondrial and nuclear DNA are commonly used methods to infer patterns of speciation. Yet, we know little about how mitochondrial and nuclear DNA interact to produce a combined-data tree. Thus, this dissertation first addresses the question of how do mitochondrial and nuclear DNA phylogenies interact to produce a combined-data tree? This question, and related questions are addressed in Chapter 1, with a thorough analysis of mitochondrial, nuclear, and combined-data trees from 14 vertebrate clades. This chapter also includes new data and a new phylogeny for the salamander genus *Plethodon,* and a discussion on why *Plethodon* seem to be an exception to the patterns uncovered in the other 13 clades.

The climatic niche of a species describes the set of precipitation and temperature conditions under which its individuals can persist and reproduce (Hutchinson 1957). The evolution of the climatic niche has previously been implicated in a variety of ecological and evolutionary subjects, for example, the impact of climatic niche conservatism (i.e., a slow rate of niche evolution) on: geographic range expansion (e.g., Wiens and Graham 2005), patterns of biogeography and species richness (e.g., Rangel et al. 2007), patterns of invasive species spread

(e.g., Thuiller et al. 2005; Mandle et al. 2010), range shifts in response to global climate change (e.g. Tingley et al. 2009), and speciation (e.g., Kozak and Wiens 2006, 2010; Smith and Beaulieu 2009; for reviews on many of these topics see Wiens and Graham 2005; Wiens et al. 2010, Peterson 2011). Smith and Beaulieu (2009) and Kozak and Wiens (2010) have suggested a relationship between the rate of climatic-niche evolution and climatic-niche breadth, although these two papers suggest opposite relationships. Neither Smith and Beaulieu (2009) nor Kozak and Wiens (2010) actually test for a relationship between rate and breadth, but infer it from results generated to answer other questions. In Chapter 2, this relationship is tested explicitly using climatic and phylogenetic data for 250 species of plethodontid salamanders.

A fundamental topic in speciation research is the different geographic modes by which new species arise, specifically, allopatric, parapatric and sympatric (Futuyma 2009; Coyne and Orr 2004). These geographic modes are based on the spatial structure of the population(s) involved (Futuyma 2009). Allopatric populations are geographically separated by an extrinsic geographic barrier (e.g., a river or mountain); parapatric populations are geographically adjacent, and a sympatric population exists in one space with no geographic separation (Futuyma 2009). The prevalence and importance of these three geographic modes has been hotly contested throughout the history of speciation research, with allopatric speciation generally thought to be the most common mode of speciation (Coyne and Orr 2004; Gavrilets 2004). It is widely accepted that species can arise in allopatry and then later become sympatrically or parapatrically distributed. In theory, patterns in the opposite direction are also possible (increasing geographic separation), but these patterns have rarely been shown. In Chapter 3, I present a multi-faceted analysis of the Eastern Red-backed Salamander, *Plethodon cinereus*, on Long Island. Previous work on this system suggested the possibility that two generally sympatric color morphs may have become parapatrically distributed on Long Island (Williams et al. 1968). My work supports this hypothesis. Additionally, I show that the pure-lead populations on eastern Long Island are divergent from other populations in morphology, ecology, and nuclear and mitochondrial genetic markers, a pattern suggesting incipient speciation. These patterns seem to be related to the different ecological preferences of the two morphs. These results suggest that spatial segregation of sympatric ecotypes might play an important part in parapatric speciation.

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Chapter 1

What are the consequences of combining nuclear and mitochondrial data for phylogenetic analysis? Lessons from *Plethodon* salamanders and 13 other vertebrate clades

Introduction

The field of molecular phylogenetics is heading towards an exciting future. In this future, genomics will allow for the use of dozens of unlinked nuclear loci to estimate phylogenies (e.g., Rokas et al. 2003; Dunn et al. 2008; Hackett et al. 2008; Wiens et al. 2008; Cibrián-Jaramillo et al 2010). These data may then be analyzed using species-tree methods that use principles of population genetics to resolve incongruence among loci (e.g., BEST, Edwards et al. 2007; STEM, Kubatko et al. 2009; *BEAST Heled and Drummond 2010).

But even as the field of phylogenetics seems to be moving towards such a future, it is clearly not there yet. For example, in animals, many phylogenies continue to be estimated based on mitochondrial (mtDNA) data alone (e.g., Abiadh et al. 2010; Byrne et al. 2010; Lavoué et al. 2010; Matsui et al. 2010), or a combined (concatenated) analysis of nuclear (nucDNA) and mtDNA data (e.g., Kozak et al. 2009; Wink et al. 2009; Ramírez et al. 2010; Roje 2010; Röll et al. 2010; San Mauro 2010). In many cases, these analyses of mtDNA or concatenated data may be necessary because sampling many species makes it impractical to include many nuclear loci (and due to fiscal constraints), and sampling many species and/or few loci makes it impractical to utilize explicit species-tree methods (despite strong theoretical justification for their use; e.g., Edwards et al. 2007; Kubatko and Degnan 2007; Heled and Drummond 2010). Many review papers have addressed the pros and cons of mtDNA data (e.g., Ballard and Whitlock 2004; Ballard and Rand 2005; Rubinoff and Holland 2005; Zink and Barrowclough 2008; Edwards and Bensch 2009), and many empirical studies have suggested the need for caution in their use (e.g., Shaw 2002; Leache 2010; Wiens et al. 2010a). However, most reviews have focused on the use of mtDNA in phylogeographic studies (e.g., Zink and Barrowclough 2008; Edwards and Bensch 2009; Barrowclough and Zink 2009) and on the question of whether mtDNA should be used in phylogenetics at all (e.g., Rubinoff and Holland 2005).

Here, we address a somewhat different question. Given that many systematists routinely estimate phylogenies using combined mtDNA and nucDNA, we ask: what are the consequences

of the common practice of combining these two types of data? For example, will the combined-data tree tend to resemble the mtDNA tree due to larger numbers of variable mtDNA characters? Or will the combined-data tree contain a mixture of clades favored by the separate data sets? Are there any generalities that can be made about when mtDNA or nucDNA data will be favored in particular clades or data sets? These questions are particularly important because many published studies simply present trees from combined analyses of mtDNA and nucDNA, without any examination of whether the mtDNA and nucDNA trees are congruent, or to what extent the combined-data tree reflects the contributions of each data set (e.g., Wink et al. 2009; Ramírez et al. 2010; Roje 2010; Röll et al. 2010; San Mauro 2010, but see for example San Mauro et al. 2004). In fact, if combined-data trees are often discordant with trees from nucDNA and largely reflect the mtDNA data instead, there may be little to be gained by collecting and adding nucDNA data in the first place (i.e., if trees are estimated from the combined-data and nucDNA have negligible impact on the combined-data analysis). To our knowledge, these important questions have never been the subject of a focused study.

In this paper, we address these and related questions, by evaluating combined-data analyses that utilize both mtDNA and nucDNA data. We approach these questions using new data and analyses for *Plethodon* salamanders, along with new analyses of existing data sets from 13 other vertebrate groups. Below, we describe the four main questions (and five associated predictions) that we address. For each of the four main questions, we are attempting to discern if there are generalities that can be made regarding the interaction of mtDNA and nucDNA data sets in a combined-data analysis.

First, are there frequent conflicts between separate mtDNA and nucDNA trees, and are the conflicting clades strongly supported by each data set? Weakly supported conflicts may be spurious and thus not problematic, whereas strongly supported conflicts may reflect more serious issues (such as long-branch attraction or discordance between gene and species trees) that may confound combined analyses (e.g., deQueiroz 1993; Bull et al 1993; de Queiroz et al 1995; Wiens 1998; Jeffroy et al. 2006; Edwards et al. 2007). As a working hypothesis, we predict that (i) discordance between mtDNA and nucDNA will generally be uncommon, and if found, will often be weakly supported by one or both data sets. This prediction is based on the simple expectation that both mitochondrial and nuclear genes will frequently share the same underlying phylogenetic history (especially given that smaller effective population sizes of mitochondrial

genes may reduce discordance due to incomplete lineage sorting; Ballard and Rand 2005), and that incongruence may often be due to estimated phylogenies that do not fully match the underlying gene trees (deQueiroz 1993; Bull et al 1993; de Queiroz et al 1995).

Second, are conflicts between the separate mtDNA and nucDNA trees generally resolved in favor of mtDNA or nucDNA in the combined-data tree? Mitochondrial genes are generally thought to evolve more rapidly than nuclear genes, and so should have more variable characters but should also have more homoplasy (e.g., Ballard and Rand 2005; Rubinoff and Holland 2005). In general, we expect conflicts between data sets to be resolved in favor of the data set with more variable characters, but also with less homoplasy. A data set with extensive conflict among characters (i.e., high homoplasy due to random noise from high overall rates of character change) may be less likely to overturn relationships inferred from a data set with less internal conflict among characters. Thus, the resolution of conflicts between mtDNA and nucDNA data sets in the combined-data tree may vary from analysis to analysis, depending on the number of characters sampled in each data set and their levels of variability and homoplasy. We predict that (ii) when mtDNA dominates a combined-data tree, it will be due to larger numbers of variable characters compared to nucDNA, and (iii) when nucDNA dominates a combined-data tree, it will be due to lower levels of homoplasy compared to mtDNA.

We address these predictions by first comparing the number of nodes shared between trees from mtDNA, nucDNA, and the combined-data, across 14 vertebrate clades. Next, we test if the proportion of nodes shared between the combined-data and mtDNA trees is correlated with the overall proportion of the variable sites in the combined data that are from mtDNA (given the prediction that the data set with more variable characters will have a stronger influence on the combined-data tree). We also test if the resolution of conflicts in the combined-data tree is related to the level of homoplasy in the mtDNA versus nucDNA data sets, given the prediction that the combined-data tree will be resolved in favor of the data set with less homoplasy (i.e., nucDNA) regardless of the relative numbers of variable sites.

Third, what generalities, if any, can we make about which nodes of the combined-data tree are resolved in favor of mtDNA vs. nucDNA? We expect that the resolution of nodes in the combined-data tree may depend on the underlying branch lengths and the depth of those branches in the tree. We predict (iv) mtDNA and nucDNA will be more congruent on longer branches, because allele histories should coalesce on longer branches, reducing discordance

among genes due to incomplete lineage sorting (Maddison 1997). Furthermore, introgression is less likely among more distantly related species (i.e., separated by longer branches), due to the accumulation of reproductive isolating mechanisms over time (Coyne and Orr 2004), which should also contribute to greater congruence between mtDNA and nucDNA on longer branches (especially if mitochondrial introgression is an important source of discordance between mtDNA and nucDNA trees; e.g., Shaw 2002). Longer branches may also be more congruent if they tend to be more strongly supported by each gene (Wiens et al. 2008), reducing spurious conflicts between mtDNA and nucDNA due to weak support. We expect shorter branches to be resolved in favor of mtDNA, given that there may be too little time for mutations to accumulate on the shortest branches for slower-evolving nuclear genes. In addition, there may be extensive incongruence among nuclear genes on short branches due to incomplete lineage sorting, also leading to weaker branch support (e.g., Wiens et al. 2008). In contrast, the mitochondrial genome is a single locus (such that there should be no incongruence among histories of mitochondrial genes), and incomplete lineage sorting may be less problematic at the between-species level due to the generally smaller effective population size of the mitochondrial genome (e.g., Moore 1995; Ballard and Whitlock 2004; Rubinoff and Holland 2005).

Finally, when mtDNA and nucDNA trees conflict, we predict (v) that nucDNA may be more likely to win conflicts deeper in the combined-data tree, while mtDNA may win resolutions that are shallower (e.g., Pereira et al. 2002; San Mauro et al. 2009). Clades deep in the tree may be harder to resolve due to long-branch attraction (Felsenstein 2004), and faster evolving genes (like mtDNA) will likely exacerbate problems of long-branch attraction (i.e., branch lengths may generally tend to be longer). The importance of tree depth may depend not only on the relative placement of branches in the tree, but also on overall branch lengths (with mtDNA being more problematic when branches are generally longer). The potential for nucDNA data to better resolve deep branches may be an important justification for including these data in the first place, along with the desire to sample unlinked loci.

In summary, a consideration of general principles suggests conflicts between mtDNA and nucDNA may be uncommon and weakly supported, and that the resolution of conflicting nodes in the combined analysis (i.e., favoring mtDNA vs. nucDNA) may vary based on the number of variable characters and level of homoplasy in each mtDNA and nucDNA data set, the lengths of

branches, and the depths of branches in the tree. We test these predictions empirically here, using new data from *Plethodon* salamanders and published data from 13 other vertebrate clades.

Plethodon is the most species-rich genus of North American salamanders (AmphibiaWeb 2012). They are terrestrial, direct-developing salamanders that are generally common and diverse in North American forests (Petranka 1998). *Plethodon* have long interested evolutionary biologists and ecologists, and hundreds of papers have been published on *Plethodon* in diverse areas, including studies of behavior, (e.g., Rollman et al. 2003; Houck et al. 2007; Deitloff et al. 2009; Kohn et al. 2009), community ecology (e.g., Hairston 1949; Adams and Rohlf 2000; Myers and Adams 2008), patterns of trait evolution (e.g., Kozak et al. 2009; Adams 2010), speciation and hybridization (e.g., Highton 1989; Hairston et al. 1992; Highton 1995; Weisrock et al. 2005; Kozak and Wiens 2006; Kozak et al. 2006; Wiens et al. 2006; Walls 2009), and response to environmental change (e.g., Walls 2009; Marsh et al. 2004; Gibbs and Karraker 2006; Vieites et al. 2007). Many of these studies have used a phylogenetic approach, making a reliable phylogeny for *Plethodon* particularly important.

Earlier studies addressed *Plethodon* phylogeny using data from allozymes (e.g., Hairston et al. 1992; Highton 1995) and mtDNA (e.g., Kozak et al. 2006), whereas more recent studies have combined mtDNA and nucDNA data (e.g., Wiens et al. 2006; Kozak et al. 2009). In general, these studies have yielded similar estimates of higher-level *Plethodon* phylogeny (e.g., most agree on a split between eastern and western species, and on the species groups in eastern North America). However, there have been substantive disagreements between studies regarding some species-level relationships (e.g., within the *cinereus* group; Kozak et al. 2009). Furthermore, all previous studies used relatively few nuclear loci (two or three; Wiens et al. 2006 Kozak et al. 2009, Vieites et al. 2007). Here we obtain new data from five nuclear loci and combine these with existing data from four nuclear genes and three mitochondrial genes, and use these data to address *Plethodon* phylogeny and general questions about combining mtDNA and nucDNA in phylogenetic studies.

Materials and Methods

Sampling of taxa and genes

We obtained DNA from 50 of the 55 currently recognized species of *Plethodon* (AmphibiaWeb 2012), representing all major clades and species groups previously recognized (e.g., Highton 1995; Wiens et al. 2006; Kozak et al. 2006; Kozak et al. 2009). Most species were represented by a single individual, but some geographically widespread species were represented by up to four individuals. We also included seven outgroup species, representing three other plethodontine genera (*Aneides, Desmognathus,* and *Ensatina*) and one genus of spelerpines (*Eurycea*). Voucher numbers and localities are listed in Online Supplement A.1. GenBank accession numbers are listed in Online Supplement A.2.

We combined mtDNA and nucDNA data from previous studies of *Plethodon* phylogeny (Kozak et al. 2006; Wiens et al. 2006; Vieites et al. 2007) with 1884 aligned base pairs (bp) of new data from five nuclear loci (572 variable characters; Table 1.1). First, we used the third intron of Rhodopsin (Rho), with primers developed specifically for use in *Plethodon* by K.H. Kozak (pers. comm.). We also tested many other nuclear introns from published lists for vertebrates (Lyons et al 1997; Friesen et al. 1997; Dolman and Phillips 2004), but found only one intron (GAPD; glyceralderhyde-3-phosphate dehydrogenase) that amplified well and was variable among *Plethodon* species. Finally, we also tested many loci (~22) from an *Ensatina* cDNA library provided by T. Devitt (pers. comm.). From this testing, we found three more introns that could be amplified in many *Plethodon* species and that were relatively variable among species. Based on BLAST searches of the sequences, these introns are associated with the nuclear genes RPL12 (60s ribosomal protein L12), ILF3 (interleukin enhancer binding factor 3) and Mlc2a (myosin light chain 2 mRNA). Primer sequences are provided in Online Supplement A.3. The length and variability of each gene are described in Table 1.1.
DNA was extracted from ethanol-preserved tissues using the Qiagen DNeasy tissue kit. Gene fragments were amplified using standard polymerase chain reaction (PCR) methods. PCR products were purified and sequenced using an ABI 3100 automated sequencer. Sequences were edited using Sequence Navigator (ver. 1.0.1, Applied Biosystems) or ContigExpress (Vector NTI build 175, Invitrogen). All sequences were initially aligned using MUSCLE (Edgar 2004), and manually refined using Se-Al v2.0a11 Carbon.

Prior to any combination of data from different genes, we used parsimony (implemented in PAUP*; Swofford 2003) to analyze each gene separately to identify any potential contaminant sequences. Contamination was hypothesized when two species had identical sequences for a given gene, and potential contaminants were re-sequenced. However, sequences were not excluded based on incongruence with previous taxonomy or with other genes, to avoid biasing the results. Only high quality sequences (i.e., few or no ambiguous bases), without potential contaminants, were used in the final analyses.

To these new data, we added 7035 bp of previously published sequence data from three sources (Table 1.1): (i) one nuclear protein-coding gene (recombination-activating gene 1; RAG-1), one nuclear intron (triose phosphate isomerase; TPI), and two protein-coding mitochondrial genes (cytochrome *b*; cyt-*b* and NADH dehydrogenase subunit 4; ND4) from Wiens et al. (2006); (ii) one mitochondrial protein-coding gene (NADH dehydrogenase subunit 2; ND2) from Kozak et al. (2009); and (iii) two nuclear protein-coding genes (proopiomelanocortin; POMC and brain-derived neurotrophic factor; BDNF) from Vieites et al. (2007). GenBank accession numbers for all previously published sequence data are provided in Online Supplement A.4.

For all newly collected data, we used the same samples from Wiens et al. (2006) and thus were able to use the same individuals to represent each species across most of the sampled mitochondrial and nuclear genes. For the other genes, we combined data from different individuals into a single terminal taxon to represent a given species. Combination of published data from different individuals generally followed Kozak et al. (2009), who carefully combined data from Kozak et al. (2006), Wiens et al. (2006), and Vieites et al. (2007).

Phylogenetic methods

Phylogenetic analyses were conducted primarily using Bayesian methods, but major results were confirmed using maximum likelihood (see below). We performed three analyses: all mitochondrial genes together, all nuclear genes together, and a combined-data analysis of all molecular data. The best-fitting model for each of the five "new" genes was identified using comparisons of the Akaike Information Criterion in MrModelTest ver. 2.0 (Nylander 2004). Given that these five genes are introns (i.e., no codons), we did not recognize partitions within these sequences. For the other genes, previous studies (e.g., Kozak et al. 2006; Wiens et al. 2006; Vieites et al. 2007) identified best-fitting models and used comparisons of Bayes factors

(Nylander et al. 2004; Brandley et al. 2005) to show that partitions based on codon positions are supported for all protein-coding loci. Models and partitions used are summarized in Table 1.1. Model parameters were unlinked between data sets. We did not assess different substitution models for different partitions within genes given that simulations show that overly simple models may be inappropriately selected when a small sample of characters is tested (Posada and Crandall 2001).

We conducted Bayesian analyses using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist 2001). For each data set, we conducted two replicate searches, each using four chains and default priors. Analyses for each data set used 6.0×10^6 generations, sampling every 1000 generations. For each analysis, we assessed when stationarity was achieved based on plots of log-likelihoods over time and on the standard deviation of split frequencies between parallel searches. In all analyses, stationarity was achieved within the first 10% of generations, and this value was used as the cut-off for burn-in (trees from the first 10% were deleted). For each analysis, the phylogeny and branch lengths were estimated from the majority-rule consensus of the pooled post burn-in trees from the two replicate searches. Clades with posterior probabilities (Pp) ≥ 0.95 were considered strongly supported (e.g., Wilcox et al. 2002; Alfaro and Zoller 2003; Erixon et al. 2003; Huelsenbeck and Rannala 2004).

Some taxa proved difficult to amplify for a given gene despite repeated attempts and development of new primers. These taxa were coded as having missing data ("?") in combined analyses. Simulations (e.g., Wiens 2003; Philippe et al. 2004; Wiens and Moen 2008) and empirical analyses (e.g., Wiens et al. 2005; Philippe et al. 2004; Driskell et al. 2004; Wiens and Morrill 2011) suggest that taxa with missing data can be accurately placed in phylogenies regardless of their number of missing data cells, especially when the total number of characters in the analysis is relatively high (and the incomplete taxa contain sufficient non-missing data). For the combined mtDNA and nucDNA sequence data (8919 characters total), each species had an average of 34.75% missing data cells, with a range among species of 0.16–72.71%. As one example, the individual with the most missing data, *P. shenandoah-2*, was placed with the other individual of *P. shenandoah* within the *cinereus* group in the combined-data analyses with strong support (Figure 1.1), suggesting that the most incomplete taxa were also accurately placed in our study. For the sake of completeness, we included data from some nuclear genes that were only sparsely sampled in previous studies (BDNF, POMC, TPI), and we did not pursue additional

sequencing of these genes ourselves (given that these genes appeared to be relatively slow evolving). Simulations suggest that adding genes with extensive missing data should generally either increase accuracy in Bayesian analyses, or else have no effect (Wiens and Morrill 2011). However, we acknowledge that these sparsely sampled genes may have less ability to help resolve conflicts between mtDNA and nucDNA.

Another concern may be that missing data impact estimates of branch lengths (but see Wiens and Morrill 2011). We tested for a relationship between the % missing data in each species and their associated, terminal branch lengths in the combined-data tree using Spearman's rank correlation in R (i.e., if missing data consistently bias branch lengths in some way, these terminal branches should be significantly longer or shorter in species with more missing data). We found no significant relationship (r_s = -0.15; P = 0.2288), suggesting that the amount of missing data had no consistent impact on estimated branch lengths.

We also ran each analysis in RAxML ver. 7.0.3 (Stamatakis 2006; 2008), conducting 100 heuristic maximum-likelihood searches combined with 500 "fastbootstrap" replicates. We used the same partitions as in the Bayesian analysis, but with the GTRGAMMA model for all partitions. This decision was made following the recommendation of Stamatakis (2008). Regardless of the initially specified model, the "fastbootstrap" setting in RAxML uses 25 rate categories (i.e., the GTRCAT model) to account for rate heterogeneity, instead of the usual four used to compute the final, optimal likelihood. Thus, a separate parameter for invariant sites should be unnecessary. The combined-data and mtDNA likelihood and Bayesian trees were nearly identical to each other (98% and 92% shared nodes, respectively). The nucDNA likelihood and Bayesian trees were less similar, but still generally concordant (78% shared nodes) and discordance was restricted to nodes with weak support (e.g., bootstrap values < 70%; Felsenstein 2004). Given the general similarity between Bayesian and likelihood results, we emphasize only the Bayesian results for simplicity.

Analyses of support and congruence among Plethodon *data sets*

We used these data to test the predictions that: (i) discordance between mtDNA and nucDNA will be uncommon and weakly supported by one or both data sets, (ii) mtDNA will dominate combined-data trees given larger numbers of variable characters, (iii) nucDNA will dominate combined-data trees due to lower homoplasy, (iv) mtDNA and nucDNA will be more

concordant on longer branches, and (v) nucDNA will dominate resolution of the combined-data tree on deeper and longer branches. Prior to conducting these analyses, outgroup taxa were pruned from all trees, as was *P. cinereus-4*, which lacked mtDNA data (otherwise, all taxa were represented in both mtDNA and nucDNA trees). All statistical analyses were conducted in R (ver. 2.11.1). Given that for all comparisons either one or both variables were not normally distributed (based on a Shapiro-Wilk test), all tests used were non-parametric unless otherwise noted.

We used the proportion of nodes shared between each pair of trees (mtDNA + nucDNA, combined-data + mtDNA, and combined-data + nucDNA) as our index of similarity between trees, based on Rohlf's (1982) consensus index (implemented in PAUP*). We also tallied the Bayesian support (posterior probability; Pp) for each concordant or discordant clade (see below). We determined if a given clade in the combined-data tree was concordant or discordant with trees from separate analyses of mtDNA and nucDNA data. We also calculated the support value (Pp) for the concordant or discordant clades. Each clade in the combined-data tree was assigned a number (Figure 1.1) and its Bayesian support (Pp) was recorded. If the same clade appeared in the separate mtDNA or nucDNA trees, it was listed as supported by that data set with a given Pp. If a clade in the combined-data tree was not present in either the mtDNA or nucDNA trees, it was considered discordant with that data set. The support value for these discordant clades was the highest Pp for any clade inconsistent with the monophyly of that combined-data clade. We then tallied the total number of shared nodes, total number of conflicting nodes, and, among those nodes in conflict, which were strongly supported ($Pp \geq 0.95$). We also recorded which data set (mtDNA or nucDNA) the strongly supported conflicts were resolved in favor of in the combined-data tree. The number of variable characters in each data set was estimated with PAUP*. The degree of homoplasy in each data set (mtDNA, nucDNA) was calculated using the consistency index (excluding uninformative characters) and the retention index (both implemented in PAUP*), with lower values for these indices indicating higher homoplasy. These values were calculated on the combined-data Bayesian tree. We recognize that these are parsimony-based estimates of homoplasy, but they nevertheless should capture variation in homoplasy relevant to all methods. While we acknowledge that model-based measures of homoplasy are potentially available, we are not aware of such a method that would allow us to readily estimate homoplasy for entire data sets of hundreds of characters.

Next, we assessed how concordance between mtDNA and nucDNA in the combined-data analysis is related to branch lengths. We assigned each branch in the combined-data tree to one of four categories: *concordant*, *mtDNA wins*, *nucDNA wins*, and *unique*. Clades in the combined-data tree congruent with separate analyses of both mtDNA and nucDNA were categorized as *concordant*. Clades in the combined-data tree congruent with the mtDNA tree but not the nucDNA tree were categorized as *mtDNA wins*. Clades in the combined-data tree congruent with the nucDNA tree but not the mtDNA tree were categorized as *nucDNA wins*. Finally, clades in the combined-data tree not congruent with either the mtDNA or nucDNA trees were categorized as *unique*. Branch lengths from the combined-data tree were used to determine the mean branch length for each category, and the difference between the means of each of the different categories was tested for significance using an exact Wilcoxon rank-sum two-sample test (equivalent to a Mann-Whitney U test). We chose to use "wilcox.exact" (package: exactRankTests) over "wilcox.test" because many of our comparisons contained ties, and the exact test calculates an exact *P*-value in the presence of ties.

We assumed that the branch lengths from the individual data sets and the combined-data tree generally reflect the true underlying branch lengths of the species tree. We confirmed that there is a significant correlation between the lengths of branches for clades shared by the mtDNA and nucDNA trees using Spearman's rank correlation ($r_s = 0.53$; $P = 0.03$), and between the lengths of the shared branches in the mtDNA and combined trees ($r_s = 0.96$; $P < 0.00001$) and the nucDNA and combined trees ($r_s = 0.73$; $P < 0.0001$). We found similar results across the other 13 clades (see below) and present these results in Online Supplement A.5.

Finally, we assessed if the combined-data tree tended to be resolved in favor of mtDNA or nucDNA at particular depths. We compared mean depth of clades between the two clade categories, *mtDNA wins* and *nucDNA wins*. We predicted that conflicts deeper in the combined-data tree would be resolved in favor of nucDNA, whereas conflicts at shallow depths would be resolved in favor of mtDNA. Clade depth was initially estimated in two ways. First, we assessed the number of nodes separating each clade from the root of the trees (e.g., clade 6 in Figure 1.1 is three nodes away from the root). Second, we summed the branch lengths (from the combined-data tree) along the shortest path from the root to the ancestor of the clade to estimate the path length. For both methods, smaller numbers are closer to the root and thus deeper, whereas larger numbers are closer to the tips, and thus more shallow. These two methods

produced strongly correlated estimates of node depth ($r_s = 0.74$; $P < 0.000001$), and in all subsequent analyses on additional data sets (see below) the first method was used to compare mean depths across categories, and is referred to as the node depth index. The difference between the means of each of the different categories was tested for significance using an exact Wilcoxon rank-sum two-sample test as described above.

Other vertebrate clades

We tested the generality of the results from *Plethodon* by conducting identical analyses on 13 other vertebrate clades: balistid fish (Dornburg et al. 2008), scarine fish (Smith et al. 2008), hemiphractid frogs (Wiens et al. 2007), hylid frogs (Wiens et al. 2005), phrynosomatid lizards (Wiens et al. 2010b), alcid birds (Pereira and Baker 2008), caprimulgid birds (Han et al. 2010), cotingid birds (Ohlson et al. 2007), dicaeid birds (Nyari et al. 2009), emydid turtles (Wiens et al. 2010a), cervid mammals (Gilbert et al. 2006), and murid rodents from both the Philippines (Jansa et al. 2006) and Sahul (Australia and New Guinea; Rowe et al. 2008). These clades were selected in order to represent the major groups of vertebrates and because they have relatively large, matched mtDNA and nucDNA data sets (see Online Supplement A.6 for data on sampling of genes and taxa, and original papers for other details). We acknowledge that these 14 clades are not a comprehensive sample of all vertebrates with published mtDNA and nucDNA data. However, each clade required extensive analyses and re-analyses (see below), and 14 clades should be adequate to detect strong general trends, if they exist (such as dominance of combined-data trees by mtDNA).

For most clades, we ran (or re-ran) Bayesian analyses to produce comparable combined-data, mtDNA, and nucDNA trees, using the same methods described for *Plethodon.* However, for emydids and phrynosomatids we used the original Bayesian results. For phrynosomatids we used results from the reduced set of 37 taxa (including *Urosaurus bicarinatus*), which have comparable data for most genes (Wiens et al. 2010b). For hylids, we used the smaller set of ~80 relatively complete taxa (Wiens et al. 2005). New analyses were run for 3 to 20 million generations, depending on the number of taxa in the data set. Any taxa in these additional data sets that were missing all of one type of data (e.g., missing all mtDNA) were removed prior to the analyses. These and other minor changes from the original methods are noted in Online Supplements A.6 and A.7. In theory, we could have done these analyses using maximum

likelihood also (or instead), but many of these data sets were initially analyzed using Bayesian methods, and previous analyses of these clades and our own experience strongly suggested that likelihood analyses would yield very similar results.

The resulting trees were subjected to the same analyses described above for *Plethodon*. In addition, we explicitly tested if mtDNA dominates combined-data trees due to a larger proportion of variable characters (prediction ii above), and if nucDNA dominates combined-data trees due to lower homoplasy (prediction iii above). For (ii), we used the proportion of the total variable sites that are derived from mtDNA data, and for (iii), we used an index of relative homoplasy (nucDNA homoplasy – mtDNA homoplasy; using both the consistency and retention indices). We correlated indices of these values with the proportion of nodes shared between the combined-data and mtDNA trees (Rohlf's consensus index values) using Pearson's product-moment correlation (note that for this analysis, all variables were normally distributed). We also used multiple regression (R package: stats; function: "lm") to test for an interaction between homoplasy and variability of data sets that may predict the proportion of nodes being shared between combined-data and mtDNA trees, once with the consistency index as our measure of homoplasy, and once with the retention index as our measure of homoplasy.

Three additional analyses of the influence of node depth were also conducted across clades. First, we tested if the overall number of sampled study clades that followed the predicted pattern (nucDNA resolves deeper nodes, mtDNA resolves shallower nodes) was significantly different from random using an exact binomial test (recommended for $n \leq 25$; Sokal and Rohlf 1995). In our case, the three potential outcomes were assigned equal probability and then lumped into two categories. The first category is those outcomes agreeing with our hypothesis: (a) nucDNA is favored deeper in the combined-data tree (smaller depth index) than mtDNA (shallower: larger depth index). The second category is those outcomes not agreeing with our hypothesis: (b) nucDNA and mtDNA are equally favored at a given depth (equal depth index) in the combined-data tree; or (c) mtDNA is favored deeper in the combined-data tree than nucDNA.

Second, because sample sizes within each of the 14 clades were sometimes small (e.g., due to a limited number of cases in which nucDNA “wins”), we pooled data across all clades. First, all node depths were standardized by dividing them by the shallowest node (largest number) in their tree to get relative node depths for each data set. For example, in the *Plethodon*

combined-data tree (Figure 1.1), node 5 is two nodes away from the root, while the shallowest node, 39, is 14 nodes away from the root, and so relative depth for node 5, is 2/14 = 0.1429. These relative node depths for each category (*mtDNA wins*, *nucDNA wins*) were pooled across clades, and the difference between the means of the two categories was tested for significance using an exact Wilcoxon rank-sum two-sample test as described above.

Finally, we tested for a relationship between node depth and branch length (given the possibility that greater congruence on deeper branches might be explained by deeper branches being longer). We tested for association between the standardized relative node depths for all nodes across all 14 clades and the corresponding standardized relative branch lengths using Spearman's rank correlation. Relative branch lengths were calculated similarly to relative node depths as described above. A clade's branch length was divided by the longest branch in the combined-data tree. For example, in *Plethodon*, the longest branch in the combined-data tree (Figure 1.1) is for node 2 at 0.0836. For Node 5, the absolute branch length is 0.0296, and its relative branch length is therefore 0.0296/0.0836, or 0.3541.

Results

Plethodon *phylogeny*

Trees from Bayesian analyses of the combined-data, mtDNA, and nucDNA for *Plethodon* are summarized in Figures 1.1, 1.2, and 1.3. The separate data sets generally agree on the major clades (eastern, western) and species groups (*cinereus, wehrlei-welleri, glutinosus*) recognized in previous studies (e.g., Highton 1995; Kozak et al. 2006; Wiens et al. 2006; Kozak et al. 2009). Nevertheless, the mtDNA and nucDNA conflict with each other at 34 of 51 nodes, and conflicts at 19 of the 34 discordant nodes are strongly supported by both data types (Table 1.2). In 15 of these 19 cases, these strongly supported conflicts are resolved in favor of the mtDNA in the combined-data tree. Of the remaining four strongly supported conflicts, three (nodes 28, 36, and 45) have topologies unique to the combined-data tree, and one (node 47) is resolved in favor of the nucDNA. The topology of the combined-data tree shares 73% of its nodes with the mitochondrial tree, and 27% with the nuclear tree (Table 1.3). The mtDNA data set has a greater number of variable characters and a higher level of homoplasy when compared to the nucDNA (Table 1.4).

The mean branch lengths and node depths grouped by clade-resolution category are summarized in Table 1.5, and significance tests are summarized in Online Supplement A.8. Concordance between the nuclear and mitochondrial trees occurs on significantly longer branches in the combined-data tree (W = 131.5; P = 0.0055). Discordance occurs at intermediate branch lengths, and the branches resolved favoring mtDNA are not significantly different in length from those favoring nucDNA clades (W = 75; P = 0.50). Clades found only in the combined-data tree are significantly shorter than clades that are concordant between mtDNA and nucDNA (W = 67; P = 0.0007) and those that are discordant (W = 104; P = 0.015). Nodes of the combined-data tree favoring the mtDNA occur at shallower depths in the combined-data tree than those favoring the nucDNA, but this trend was not significant (W = 80; P = 0.3454).

Comparisons across clades

Trees from Bayesian analyses of the combined-data, mtDNA, and nucDNA for the other 13 vertebrate clades are summarized in Online Supplement A.9. Combining our results from *Plethodon* with those from these 13 other clades, we find that discordance between trees from mtDNA and nucDNA is very common, with only 30–70% (mean = 49%) of nodes concordant in each study. Seven of the 14 data sets show extensive incongruence between mtDNA and nucDNA, with only a minority of nodes (range among seven data sets = 30–49%; mean = 38%; Table 1.2) in common between them in each data set. In addition, four of the remaining seven data sets show only a slight majority of congruent nodes between mtDNA and nucDNA (range among four data sets = 54–58%; mean = 56%; Table 1.2). The final three data sets show more extensive congruence (range among three data sets = 63–70%; mean = 67%; Table 1.2).

Nevertheless, despite this widespread incongruence, in all clades except *Plethodon*, only a minority of the conflicts between mtDNA and nucDNA are strongly supported (range among 13 clades = 9–44%; mean = 25%; *Plethodon* = 56%; Table 1.2). These strongly supported conflicts are often resolved in favor of mtDNA (mean = 56% across the 14 data sets; 79% in *Plethodon*), but the trend is not significant for most data sets, and in four out of 14 data sets, these strong conflicts are more often resolved in favor of nucDNA (Table 1.2). Of the remaining conflicts, 0–46% (mean = 26%) were weakly supported by both data sets, 0–56% (mean = 23%) were strongly supported by nucDNA, but weakly supported by mtDNA, and 0–44% (mean = 24%) were weakly supported by nucDNA, but strongly supported by mtDNA (Table 1.2).

Surprisingly, we find that the combined-data trees are more similar to the nucDNA trees for eight of 14 data sets (Table 1.3). Four of those eight data sets have nearly equal numbers of variable characters between the mtDNA and nucDNA data sets (balistid fish, cotingid birds, emydid turtles, murid rodents (Philippines); Table 1.4), but two actually have many more variable mtDNA characters than nucDNA characters (hylid frogs, phrynosomatid lizards; Table 4). The remaining two data sets (caprimulgid birds, murid rodents (Sahul = Australia and New Guinea); Table 1.4), had substantially more variable nucDNA characters than mtDNA characters.

The ability of nucDNA data to sometimes dominate more nodes of the combined-data tree with only a minority of variable characters is surprising. One obvious explanation for this pattern is that the mtDNA characters have consistently higher levels of homoplasy than nucDNA characters (Table 1.4). However, the proportion of shared nodes between the combined-data tree and the mtDNA tree (first column, Table 1.3) was not correlated with either of our indices of relative mtDNA homoplasy (consistency index: $r = 0.33$; $P = 0.26$; retention index: $r = 0.19$; $P = 0.51$). The proportion of shared nodes between the combined-data tree and the mtDNA tree was not significantly correlated with the proportion of mtDNA variable sites ($r = 0.49$; $P = 0.08$), although there is a trend in this direction. Multiple regression of the proportion of nodes shared between the mtDNA and combined-data trees on homoplasy and variability was not significant for either homoplasy index (all values of $P \geq 0.807$).

Comparisons across all 14 data sets confirm our prediction that branches in the combined-data tree that are concordant between mtDNA and nucDNA are longer on average than other branches (Table 1.5; Figure 1.4; *concordant* vs. *discordant* in Online Supplement A.8). However, contrary to our expectations, there is no support for the hypothesis that shorter branches tend to be resolved in favor of mtDNA and longer branches in favor of nucDNA (see Online Supplement A.8). The only significant pattern is found in hylid frogs ($W = 37$; $P = 0.0475$) and caprimulgid birds ($W = 91$; $P = 0.0011$), in which clades resolved in favor of mtDNA are significantly longer than those resolved in favor of nucDNA (the opposite of our expectations).

Thirteen out of 14 clades (all except hylids) show the predicted pattern in which deeper branches of the combined-data tree are resolved in favor of nucDNA and shallower branches are resolved in favor of mtDNA (Table 1.5; Online Supplement A.8). Although this pattern is only

significant within hemiphractids ($W = 69$; $P = 0.0055$), finding the same pattern in 13 of 14 clades is statistically significant ($P << 0.0001$; exact binomial test). The lack of significant patterns within each clade may reflect limited sample size for significance testing (e.g., phrynosomatids have only two clades resolved in favor of mtDNA). Pooling relative node depths across clades shows that branches on which mtDNA is favored are significantly shallower than branches on which nucDNA is favored ($W = 5655.5$; $P = 0.0133$; Figure 1.4), and nodes that are concordant between mtDNA and nucDNA are significantly deeper than discordant clades (W=37282.5; P=0.0261; Figure 1.5). Across all clades, relative node depth is negatively correlated with relative branch length ($r_s = -0.31$; $P << 0.00001$), such that longer branches tend to be found deeper in the tree. The longer branches deeper in the tree may explain the greater concordance between mtDNA and nucDNA on deep branches.

Discussion

Consequences of combining mitochondrial and nuclear data for phylogenetic analysis

Combining data from nucDNA and mtDNA is a common practice in phylogenetic studies, but one whose consequences have gone largely unstudied (or at least under-reported). This is surprising given the extensive debate about pros and cons of mtDNA data for phylogenetic analysis (e.g., Moore 1995; Ballard and Whitlock 2004; Ballard and Rand 2005; Rubinoff and Holland 2005; Zink and Barrowclough 2008; Edwards and Bensch 2009; Barrowclough and Zink 2009), and about combining data in general (e.g., Bull et al. 1993; de Queiroz et al. 1995; Wiens 1998; Degnan and Rosenberg 2006; Edwards et al. 2007; Kubatko and Degnan 2007). In this study, we test several key predictions about how mtDNA and nucDNA interact in combined-data analyses, using new data from *Plethodon* salamanders and published data from 13 other vertebrate clades.

Our results suggest that even though conflicts between mtDNA and nucDNA are widespread in these 14 groups, the general dominance of mtDNA in combined-data trees is not supported, even in two clades in which the number of variable mtDNA characters greatly outnumbers those from the nucDNA (see below). We find that discordance between mtDNA and nucDNA trees is common: across the 14 data sets, 30–70% (mean = 49%) of nodes are concordant. This suggests that the issue of how these conflicts are resolved in the combined-data

analysis is of critical importance. But we also find that many of these conflicts are only weakly supported by one or both data sets. Strongly supported conflicts (for which conflicting clades are strongly supported by each type of data) tend to be uncommon (mean = 27% of discordant nodes, range 9–56%), and may be resolved in favor of either mtDNA or nucDNA with almost equal frequency (mean = 54% in favor of mtDNA, range = 0-100%).

Surprisingly, we find that in the majority of the 14 data sets, the combined-data tree is more similar to the nucDNA tree than the mtDNA tree (i.e., shares more nodes). In fact, nucDNA can dominate the combined-data tree even when the number of variable mtDNA characters is 2–3 times that of the variable nucDNA characters (i.e., in hylid frogs and phrynosomatid lizards). The most obvious explanation for this pattern is that the lower homoplasy of nucDNA characters may outweigh the influence of the larger numbers of variable mtDNA characters. However, our analyses of the relationship between homoplasy levels and the dominance of the combined-data tree by mtDNA do not support the idea that more homoplasy in mtDNA necessarily leads to combined-data trees that more closely resemble the nucDNA trees. There are several possible explanations for this unexpected combination of results. One is that the differences in homoplasy between mtDNA and nucDNA are primarily what matter, and that variation in levels of homoplasy among mtDNA data sets (which is what our indices mostly reflect, see Materials and Methods) is relatively unimportant. Another (non-exclusive) possibility is that the conflicts between mtDNA and nucDNA occur because of processes that are not reflected by levels of homoplasy in the mtDNA data (e.g., introgression, incomplete lineage sorting).

Contrary to our expectations, we find no evidence that shorter branches are generally resolved in favor of mtDNA. In fact, among the 14 data sets, the only significant trend is for longer branches to be resolved in favor of mtDNA, which occurs in hylid frogs and caprimulgid birds. We do find that within a given combined-data tree, there is a tendency for longer branches to be agreed upon by mtDNA and nucDNA. This result parallels the pattern seen among nuclear genes in some studies, where congruence between genes increases on longer branches, possibly due to fewer conflicts between gene and species trees associated with incomplete lineage sorting (e.g., Wiens et al. 2008; Wiens et al. 2010a). The causes of discordance between mtDNA and nucDNA on shorter branches are not entirely clear. Most of the conflicts (73%) we uncovered between mtDNA and nucDNA are not strongly supported by one or both data sets. Therefore,

spurious resolution of weakly supported clades may be a major cause of disagreement. We also find that clades that are absent in both the separate mtDNA and nucDNA trees (*unique*) tend to be the shortest branches in the combined-data tree, suggesting that they have few supporting characters from either data set.

Finally, our prediction that deeper nodes tend to be resolved in favor of nucDNA was supported in 13 out of 14 data sets, and when data were pooled across clades. Interestingly, one clade (hylid frogs) showed the opposite pattern, with deeper nodes typically resolved in favor of mtDNA. In fact, the idea that mtDNA and nucDNA will resolve different portions of the phylogeny (shallow vs. deep; e.g., Pereira et al. 2002; San Mauro et al. 2009) may be one of the major motivations for obtaining and combining these data types in the first place. Our prediction was based on the idea that long-branch attraction might be more common among deeper nodes, and that slow-evolving nucDNA might help resolve such problems. This prediction is further supported by a significant negative correlation between branch length and node depth, suggesting that longer branches are indeed found deeper in the tree (note that without considerable rate heterogeneity it would be difficult for a long branch to be shallowly placed). Our results here suggest that nucDNA does indeed help to resolve deeper branches in the phylogeny (see also Pereira et al. 2002; San Mauro et al. 2009), and for this reason, nucDNA data are worth pursuing in clades for which phylogeny was previously estimated by mtDNA only.

In summary, our results suggest that combined analyses of mtDNA and nucDNA are not necessarily dominated by mtDNA, even though conflicts between mtDNA and nucDNA are indeed common. Thus, both data sets typically contribute to resolution of combined-data trees, and the addition of nucDNA data can be worthwhile. However, we do find considerable variation in these patterns among clades, which suggests the need for routine checking of incongruence between mtDNA and nucDNA and its impacts on combined analyses. For example, our results for *Plethodon* show widespread, strongly-supported incongruence between mtDNA and nucDNA that is generally resolved in favor of mtDNA (despite inclusion of nine nuclear genes). It should also be noted that we only considered data sets in which the overall taxon sampling of mtDNA and nucDNA was basically identical. Cases in which one data set is more broadly sampled might certainly alter these dynamics (e.g., nucDNA for 80 species and mtDNA for ~200 species; Wiens et al. 2005). Furthermore, dramatic differences in sampling of

genes between these genomes could obviously influence the results (e.g., whole mitochondrial genomes vs. a single nuclear gene; San Mauro et al. 2004). Nevertheless, our results provide an initial baseline for understanding how mtDNA and nucDNA may typically interact to determine the results of combined analyses.

Plethodon *phylogeny*

Our survey of vertebrate clades shows that the results for *Plethodon* are quite unusual, in both the preponderance of widespread, strongly supported incongruence between mtDNA and nucDNA, and the consistency with which the incongruence is resolved in favor of the mtDNA. We speculate that mitochondrial introgression between young but distantly related species may be a major factor driving this pattern. For example, *P. shermani* has been previously classified as a member of the *jordani* species complex (e.g., Highton and Peabody 2000). All members of the *jordani* complex, except *P. shermani*, are placed in clade B in the combined-data tree (Figure 1.1). We find *P. shermani* in clade A in the mtDNA (Figure 1.2) and combined-data (Figure 1.1) trees, where it is placed in a clade with *P. aureolus*, with which it is known to hybridize (Highton 1995; Weisrock et al. 2005; Wiens et al. 2006). In contrast, in the nucDNA tree (Figure 1.3), *P. shermani* is placed in clade B with strong support. This pattern suggests the possibility that *P. shermani* belongs to clade B, but mitochondrial introgression with *P. aureolus* leads to its placement in clade A in the mtDNA and combined-data trees. Placement of this species into these two different major clades by mtDNA and nucDNA contributes to the broad-scale incongruence between these data sets.

Despite the widespread incongruence between mtDNA and nucDNA, we find some cases where the new nucDNA data do appear to improve the combined-data results. For example, in the mtDNA tree (Figure 1.2), *P. jordani* and *P. metcalfi* (of the *jordani* complex) are at the base of the *glutinosus* group, while the rest of the *jordani* complex (*P. amplus, P. cheoah, P. meridianus, P. montanus*) is within clade B (except for *P. shermani,* see above). In the nucDNA (Figure 1.3) and combined-data (Figure 1.1) analyses in the present study, *P. jordani* and *P. metcalfi* are placed in clade B with strong support.

Despite these potential improvements, there are still many issues to be resolved with future work on *Plethodon* systematics. Many clades in the nucDNA tree (Figure 1.3) are still weakly supported (despite use of nine nuclear genes), especially in the rapid, recent radiation of

the *glutinosus* complex. Sequencing yet more nuclear loci may be helpful here. There also appear to be important taxonomic issues to resolve in the *glutinosus* complex, which will require sampling many populations as well as many loci. For example, individuals of *P. aureolus* and *P. glutinosus* are found in separate clades in both mtDNA and nucDNA, suggesting the presence of multiple species. Sampling the same nuclear genes used here in individuals from many localities within the range of each species may be a useful next step for better resolving both species limits and the phylogeny.

Conclusions

Combined analyses of mtDNA and nucDNA are common, but the consequences of combining these data are largely unexplored. This trend is somewhat unsettling given that use of mtDNA is somewhat controversial, and given the possibility that mtDNA might dominate combined analyses due to larger numbers of variable characters. Our results here for 14 vertebrate clades show that even though conflicts between mtDNA and nucDNA are indeed widespread, they are typically weakly supported, and mtDNA does not dominate combined-data trees in the majority of clades. Instead, both data types often contribute to resolving the combined-data tree, with nucDNA being particularly useful for deep branches. Thus, even though nucDNA data is traditionally more difficult to obtain in animals than mtDNA (hence the large number of studies still using mtDNA alone), and typically yields fewer variable characters per base pair (Table 1.4), our results suggest that the added cost and effort needed to obtain and add nucDNA is not necessarily wasted in a combined analysis. However, our new results for *Plethodon* show that, even with large numbers of nuclear loci, mtDNA may still dominate a combined-data tree. Therefore, testing for the congruence of mtDNA and nucDNA and the impact of each data set on combined analyses is an essential precaution.

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Table 1.1. Genes used in the phylogenetic analysis of *Plethodon.*

Type of locus	**Locus**	**Length**	**Variable characters**	**Parsimony-informative characters**	**Best-fitting model**	**Partitions**	**Number of taxa sampled**	**Data source**
Nuclear introns	GAPD	659	221	92	GTR + Γ	none	48	this study
	ILF3	281	56	34	HKY	none	40	this study
	Mlc2a	257	79	39	GTR + Γ	none	55	this study
	RPL12	463	159	90	HKY + Γ	none	48	this study
	RHO	224	57	43	HKY + I	none	62	this study
	TPI	1938	569	211	GTR + Γ	intron/exon	29	Wiens et al. 2006
Nuclear exons	RAG-1	1467	358	248	GTR + I + Γ	codon	60	Wiens et al. 2006
	BDNF	707	67	29	GTR + Γ	codon	14	Vieites et al. 2007
	POMC	481	98	41	GTR + Γ	codon	15	Vieites et al. 2007
Mitochondrial genes	Cyt-*b*	649	369	313	GTR + I + Γ	codon	66	Wiens et al. 2006
	ND4	686	409	364	GTR + I + Γ	codon	65	Wiens et al. 2006
	ND2	1107	741	635	GTR + I + Γ	codon, tRNA-TRP	52	Weisrock et al. 2005

Table 1.2. Congruence between mtDNA, nucDNA, and combined-data trees. Each column reports the percentage of total nodes (second column) that fall into the following categories: (a) **concordant nodes** are those present in the combined-data tree that are also present in both mtDNA and nucDNA trees; (b) **discordant nodes** are absent in one or both of the trees from the separate data sets; (c) **strongly supported discordance** indicates branches for which conflicting resolutions in mtDNA and nucDNA are strongly supported (Pp ≥ 0.95) by each one; (d) **weak mtDNA (or nucDNA), strong nucDNA (or mtDNA)** indicates branches for which conflicting resolutions were weakly supported by one (mtDNA or nucDNA) and strongly supported by the other; (e) **weakly supported discordance** indicates branches for which conflicting resolutions in mtDNA and nucDNA are weakly supported by both; (f) the far-right column gives the proportion of nodes with strongly supported discordance that are resolved in favor of the mtDNA in the combined-data tree. *P*-values indicate whether the number of these resolutions favoring mtDNA data are significantly different from random (exact binomial, *p* = 0.50). Significant *P*-values are bold faced.

Clade	Total nodes	Concordant nodes	Discordant nodes	Strongly supported discordance	Weak mtDNA, strong nucDNA	Strong mtDNA, weak nucDNA	Weakly supported discordance	Strong discordance resolved favoring mtDNA
Balistid fish	23	39%	61%	14%	43%	21%	21%	50% mtDNA, *P* = 0.5000
Scarine fish	40	55%	45%	44%	22%	17%	17%	75% mtDNA, *P* = 0.1094
Hemiphractid frogs	40	45%	55%	36%	18%	23%	23%	75% mtDNA, *P* = 0.1094
Hylid frogs	76	54%	46%	17%	29%	9%	46%	67% mtDNA, *P* = 0.2344
Plethodon salama	51	33%	67%	56%	21%	24%	0%	79% mtDNA,

nders								**_P_ = 0.0074**
Phrynosomatid lizards	35	49%	51%	28%	44%	17%	11%	0% mtDNA, **_P_ = 0.0313**
Alcid birds	21	67%	33%	29%	14%	43%	14%	100% mtDNA, _P_ = 0.2500
Caprimulgid birds	55	56%	44%	17%	4%	33%	46%	50% mtDNA, _P_ = 0.3750
Cotingid birds	35	63%	37%	23%	31%	0%	46%	67% mtDNA, _P_ = 0.3750
Dicaeid birds	28	36%	64%	17%	11%	39%	33%	33% mtDNA, _P_ = 0.3750
Emydid turtles	36	33%	67%	29%	33%	17%	21%	38% mtDNA, _P_ = 0.2734
Cervid mammals	23	30%	70%	19%	0%	56%	25%	67% mtDNA, _P_ = 0.3750
Murid rodents (Philippines)	55	58%	42%	9%	43%	13%	35%	50% mtDNA, _P_ = 0.5000
Murid rodents (Sahul)	60	70%	30%	39%	22%	17%	22%	0% mtDNA, **_P_ = 0.0078**

Table 1.3. Similarity between trees from different data sets. The proportion of nodes shared between each pair of trees (mtDNA, nucDNA and combined-data) for each clade. Boldfaced proportion indicates which of the two data sets (mtDNA, nucDNA) the combined-data tree is more similar to.

Clade	Combined-data and mtDNA	Combined-data and nucDNA	mtDNA and nucDNA
Balistid fish	16%	**24%**	11%
Scarine fish	**83%**	63%	50%
Hemiphractid frogs	**64%**	52%	30%
Hylid frogs	27%	**44%**	13%
Plethodon salamanders	**73%**	27%	23%
Phrynosomatid lizards	37%	**71%**	26%
Alcid birds	**91%**	33%	23%
Caprimulgid birds	53%	**82%**	38%
Cotingid birds	53%	**71%**	35%
Dicaeid birds	**80%**	34%	24%
Emydid turtles	54%	**60%**	37%
Cervid mammals	**99%**	37%	27%
Murid rodents (Philippines)	30%	**63%**	23%
Murid rodents (Sahul)	55%	**96%**	53%

Table 1.4. Variability and homoplasy in each type of data. A summary of the number of variable characters that each data type (mtDNA, nucDNA) contributes to each combined analysis and the amount of homoplasy in each data set (lower values indicate more homoplasy; see Online Supplement A.6 for additional details on each data set). The consistency index excludes uninformative characters. Outgroups were not included.

Clade	nucDNA variable characters	mtDNA variable characters	Ratio of variable characters nucDNA: mtDNA	nucDNA consistency index / retention index	mtDNA consistency index / retention index
Balistid fish	341	337	1.01	0.5851 / 0.7298	0.4175 / 0.5380
Scarine fish	612	743	0.82	0.5579 / 0.7874	0.3805 / 0.6012
Hemiphractid frogs	441	1344	0.33	0.6427 / 0.8552	0.3065 / 0.4589
Hylid frogs	715	1442	0.50	0.2844 / 0.5646	0.1486 / 0.3135
Plethodon salamanders	1204	1400	0.86	0.6042 / 0.8132	0.3069 / 0.6329
Phrynosomatid lizards	1155	2258	0.51	0.5498 / 0.7490	0.3327 / 0.3284
Alcid birds	255	1559	0.16	0.6471 / 0.7918	0.4129 / 0.5823
Caprimulgid birds	790	522	1.51	0.4708 / 0.7560	0.2216 / 0.5078
Cotingid birds	440	493	0.89	0.5858 / 0.7162	0.2300 / 0.3154
Dicaeid birds	86	660	0.13	0.8043 / 0.9455	0.7067 / 0.3793
Emydid turtles	477	460	1.04	0.6581 / 0.8662	0.4591 / 0.7820
Cervid mammals	127	624	0.20	0.7414 / 0.9085	0.3097 / 0.4704
Murid rodents (Philippines)	640	628	1.02	0.4179 / 0.6474	0.1649 / 0.3342
Murid rodents (Sahul)	4226	1175	3.60	0.5094 / 0.7129	0.1666 / 0.2974

Table 1.5. Mean branch lengths and node depths across clade resolution categories. A summary of the mean branch lengths and mean node depths of branches in the combined-data trees for each clade, grouped by how they are resolved. For the node depths, larger numbers indicate shallower nodes (i.e. those closer to the tips and farther from the root). The last row of pooled data reports mean relative branch lengths and mean relative node depths. Significance testing is summarized in the Results and more extensively in Online Supplement A.8.

Clade	**Type of node**	**Number of nodes**	**Mean branch length**	**Standard error branch lengths**	**Mean node depth**	**Standard error node depth**
Balistid fish	*Concordant*	9	0.0113	0.0024	6.44	0.53
	mtDNA wins	3	0.0045	0.0021	6.33	0.84
	nucDNA wins	5	0.0042	0.0004	6.00	0.88
	Unique	6	0.0051	0.0005	3.00	0.73
Scarine fish	*Concordant*	22	0.0110	0.0028	5.68	0.53
	mtDNA wins	10	0.0042	0.0040	6.90	0.95
	nucDNA wins	4	0.0058	0.0008	5.75	1.00
	Unique	4	0.0023	0.0007	6.75	1.97
Hemiphractid frogs	*Concordant*	18	0.0528	0.0129	5.72	0.67
	mtDNA wins	13	0.0118	0.0019	8.85	1.36
	nucDNA wins	6	0.0142	0.0022	4.67	0.49
	Unique	3	0.0068	0.0007	8.00	1.00
Hylid frogs	*Concordant*	41	0.0828	0.0081	10.12	0.63
	mtDNA wins	11	0.0686	0.0049	7.45	0.86
	nucDNA wins	13	0.0299	0.0150	9.46	1.11
	Unique	11	0.0196	0.0024	9.27	1.02
Plethodon salamanders	*Concordant*	17	0.0199	0.0054	5.00	0.74
	mtDNA wins	25	0.0067	0.0020	8.32	1.93
	nucDNA wins	5	0.0052	0.0011	6.20	0.67
	Unique	4	0.0023	0.0003	8.25	1.11
Phrynosomatid lizards	*Concordant*	17	0.1046	0.0169	5.29	0.73
	mtDNA wins	2	0.0221	0.0126	8.00	1.31
	nucDNA wins	12	0.0395	0.0091	7.92	3.00
	Unique	4	0.0152	0.0032	9.50	2.63
Alcid birds	*Concordant*	14	0.0478	0.0093	3.71	0.40
	mtDNA wins	6	0.0212	0.0073	3.17	0.65
	nucDNA wins	1	0.0057	-	2.00	-
	Unique	0	-	-	-	-
Caprimulgid birds	*Concordant*	31	0.0562	0.0085	7.87	0.54
	mtDNA wins	10	0.0309	0.0042	8.40	0.99
	nucDNA wins	10	0.0149	0.0045	7.00	1.06
	Unique	4	0.0090	0.0023	8.00	0.82
Cotingid birds	*Concordant*	22	0.0550	0.0081	5.27	0.52
	mtDNA wins	5	0.0132	0.0017	5.60	1.03
	nucDNA wins	7	0.0140	0.0029	5.29	1.15

	Unique	1	0.0091	-	1.00	-
Dicaeid birds	*Concordant*	10	0.0919	0.0104	3.70	0.37
	mtDNA wins	13	0.0604	0.0042	5.54	1.49
	nucDNA wins	4	0.0312	0.0162	5.25	0.79
	Unique	1	0.0214	-	5.00	-
Emydid turtles	*Concordant*	12	0.0078	0.0013	3.50	0.47
	mtDNA wins	7	0.0024	0.0004	6.29	0.88
	nucDNA wins	12	0.0032	0.0006	6.00	0.87
	Unique	5	0.0007	0.0002	7.80	1.39
Cervid mammals	*Concordant*	7	0.0288	0.0030	2.57	0.43
	mtDNA wins	14	0.0160	0.0022	4.36	0.34
	nucDNA wins	2	0.0066	0.0014	4.00	3.00
	Unique	0	-	-	-	-
Murid rodents (Philippines)	*Concordant*	32	0.1341	0.0146	7.06	0.50
	mtDNA wins	7	0.1011	0.0083	6.71	0.89
	nucDNA wins	13	0.0494	0.0644	6.46	1.15
	Unique	3	0.0305	0.0065	3.67	1.20
Murid rodents (Sahul)	*Concordant*	42	0.0097	0.0013	6.64	1.03
	mtDNA wins	4	0.0018	0.0003	9.50	4.75
	nucDNA wins	13	0.0038	0.0008	6.77	1.88
	Unique	1	0.0015	-	7.00	-
Pooled across clades	*Concordant*	294	0.0556	0.0035	0.51	0.01
	mtDNA wins	130	0.0265	0.0045	0.60	0.02
	nucDNA wins	107	0.0198	0.0024	0.52	0.02
	Unique	47	0.0108	0.0015	0.53	0.04

Figure 1.1: Combined-data *Plethodon* phylogeny. Phylogeny of the salamander genus *Plethodon* based on a combined, partitioned Bayesian analysis of mitochondrial DNA (mtDNA) and nuclear DNA (nucDNA). An asterisk next to a node indicates strong support ($Pp \geq 0.95$). Small open circles on a node indicate $Pp < 0.95$, and these values are listed. Integers next to each node correspond to clade numbers used in analyses of congruence and discordance. A clade was not numbered if all terminal taxa belong to the same species. The outgroup taxa are excluded (but only from the figure) to facilitate presentation of branch lengths, and the root is indicated with a large open circle.

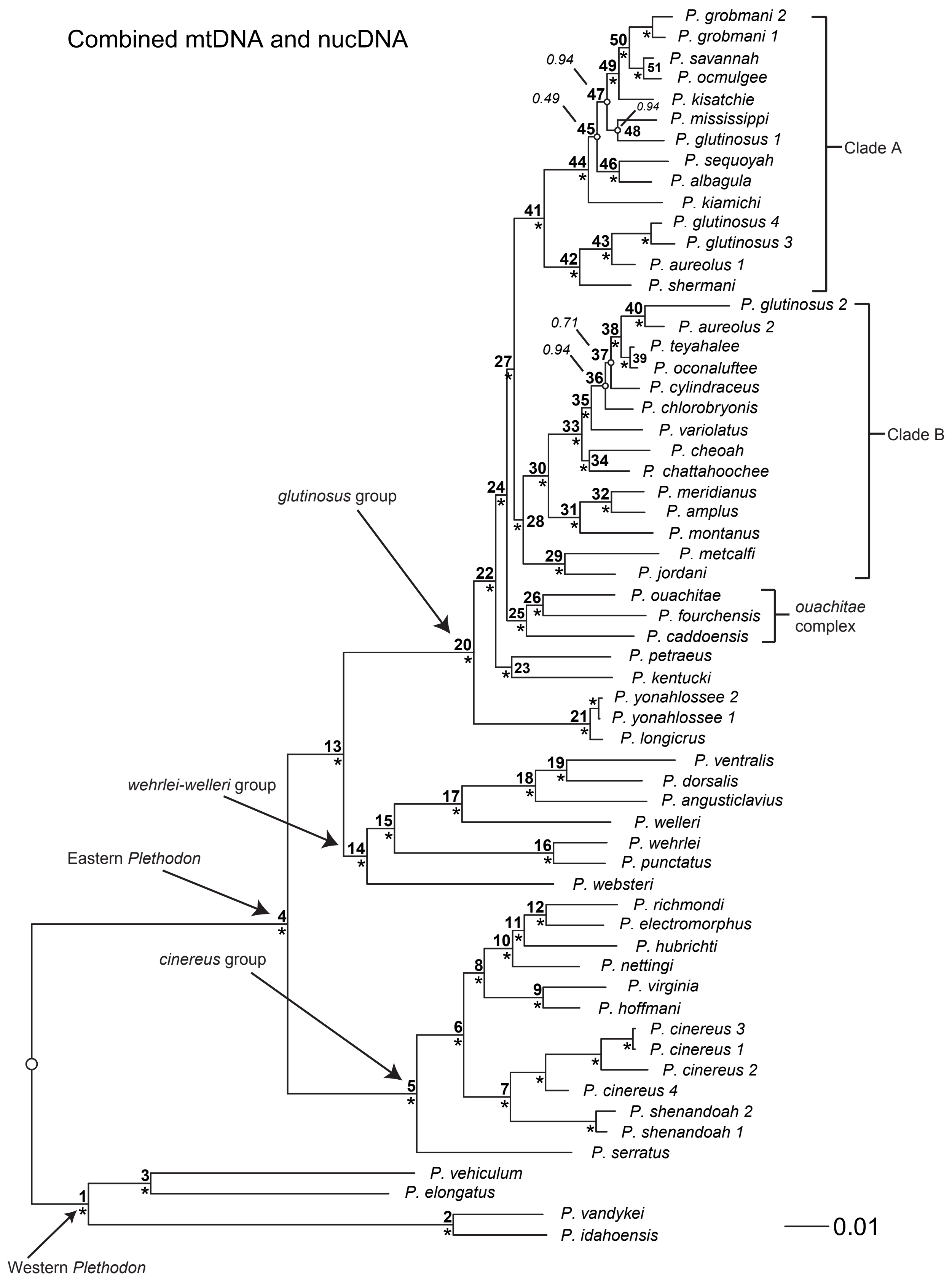

Combined mtDNA and nucDNA
P. grobmani 2
P. grobmani 1
P. savannah
P. ocmulgee
P. kisatchie
P. mississippi
P. glutinosus 1
P. sequoyah
P. albagula
P. kiamichi
P. glutinosus 4
P. glutinosus 3
P. aureolus 1
P. shermani
Clade A
P. glutinosus 2
P. aureolus 2
P. teyahalee
P. oconaluftee
P. cylindraceus
P. chlorobryonis
P. variolatus
P. cheoah
P. chattahoochee
P. meridianus
P. amplus
P. montanus
P. metcalfi
P. jordani
Clade B
P. ouachitae
P. fourchensis
P. caddoensis
ouachitae complex
P. petraeus
P. kentucki
P. yonahlossee 2
P. yonahlossee 1
P. longicrus
glutinosus group
P. ventralis
P. dorsalis
P. angusticlavius
P. welleri
P. wehrlei
P. punctatus
P. websteri
wehrlei-welleri group
Eastern Plethodon
P. richmondi
P. electromorphus
P. hubrichti
P. nettingi
P. virginia
P. hoffmani
P. cinereus 3
P. cinereus 1
P. cinereus 2
P. cinereus 4
P. shenandoah 2
P. shenandoah 1
P. serratus
cinereus group
P. vehiculum
P. elongatus
P. vandykei
P. idahoensis
Western Plethodon
0.01
0.94
0.49
0.71

Figure 1.2: Mitochondrial *Plethodon* phylogeny. Phylogeny of the salamander genus *Plethodon* based on a combined, partitioned Bayesian analysis of mitochondrial DNA (mtDNA) only. An asterisk next to a node indicates strong support (Pp ≥ 0.95). Small open circles on a node indicate Pp < 0.95, and these values are listed. The outgroup taxa are excluded (but only from the figure) to facilitate presentation of branch lengths, and the root is indicated with a large open circle.

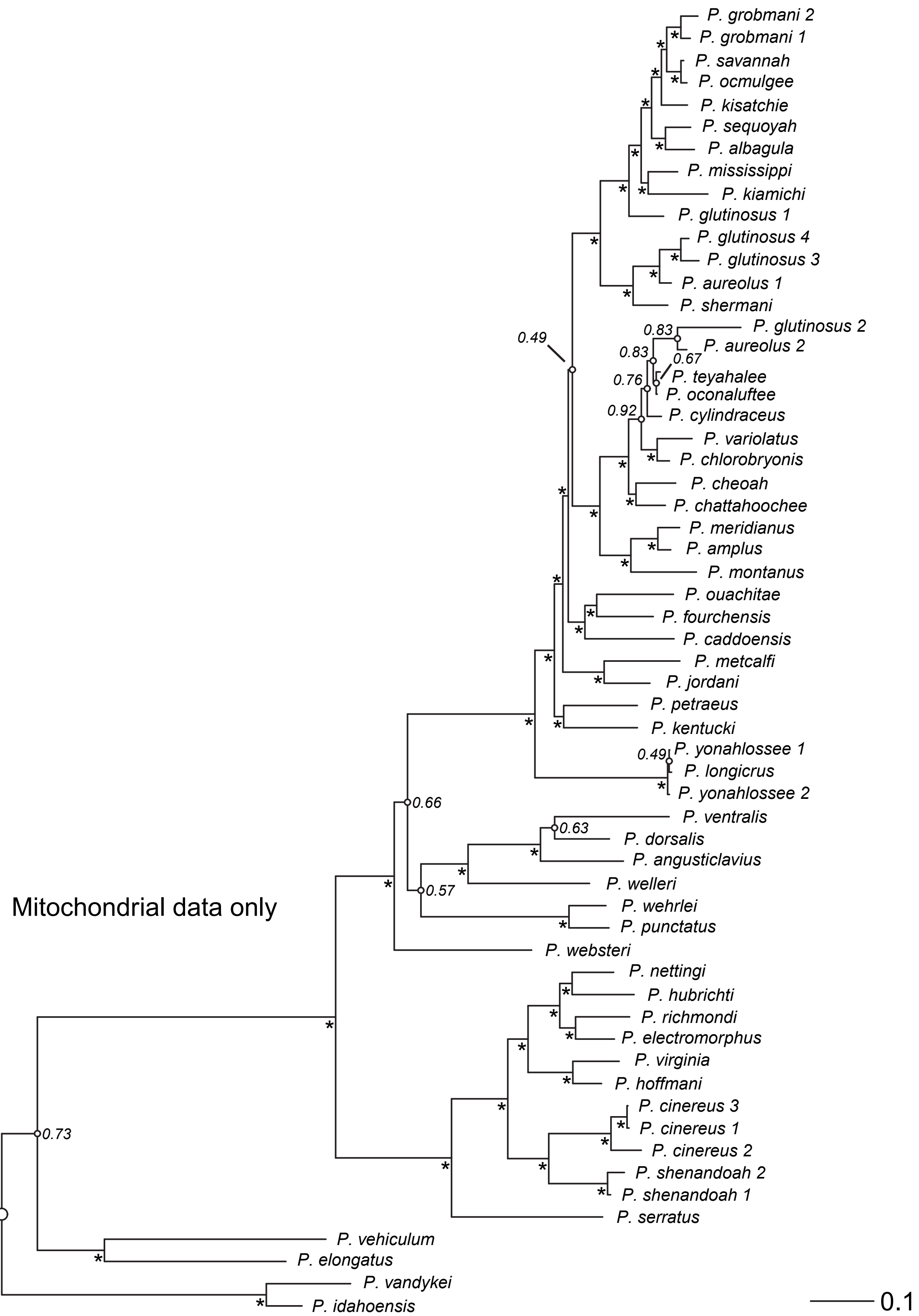
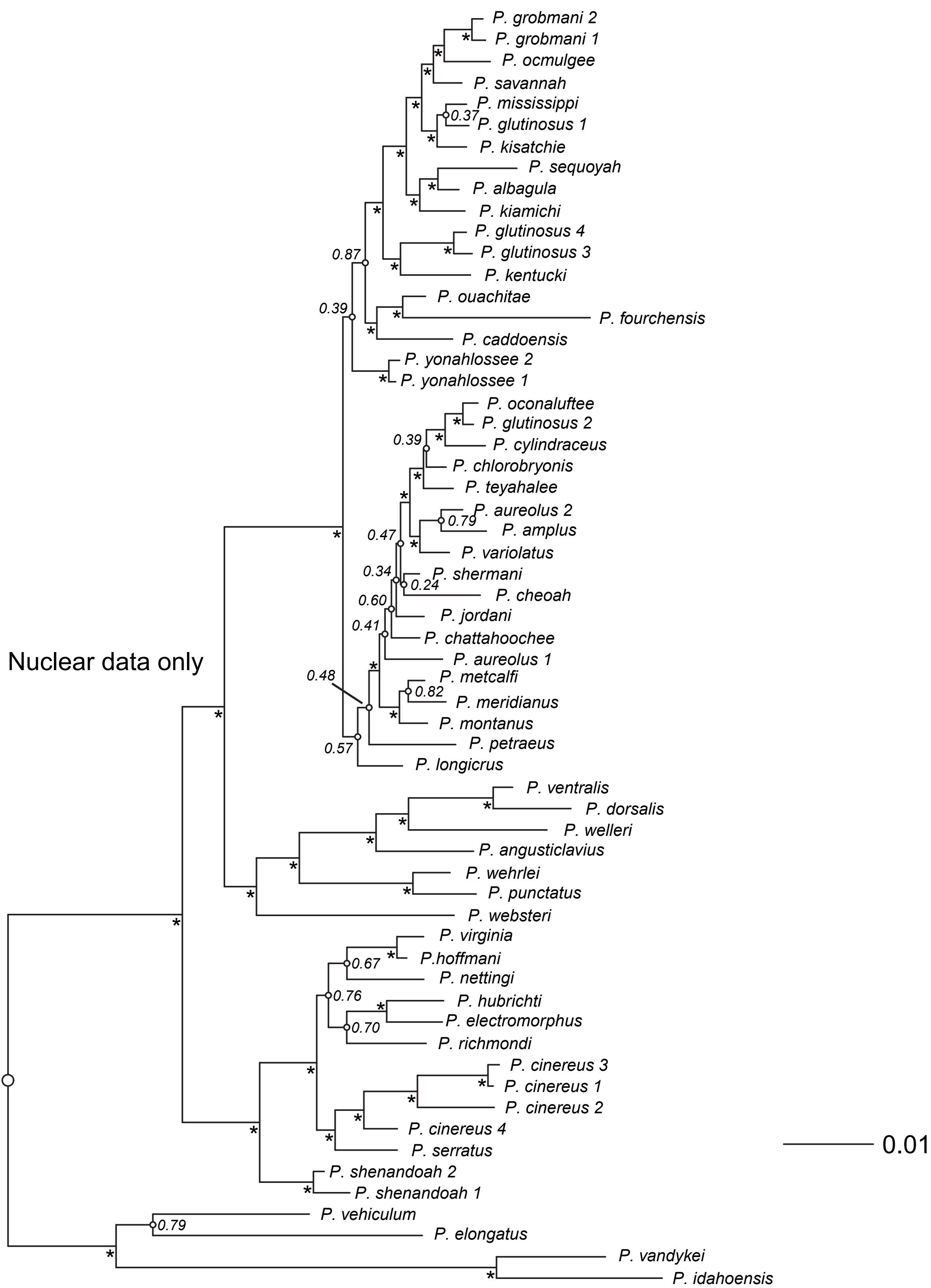

P. grobmani 2
P. grobmani 1
P. savannah
P. ocmulgee
P. kisatchie
P. sequoyah
P. albagula
P. mississippi
P. kiamichi
P. glutinosus 1
P. glutinosus 4
P. glutinosus 3
P. aureolus 1
P. shermani
0.83
P. glutinosus 2
0.49
0.83
P. aureolus 2
0.67
P. teyahalee
0.76
P. oconaluftee
0.92
P. cylindraceus
P. variolatus
P. chlorobryonis
P. cheoah
P. chattahoochee
P. meridianus
P. amplus
P. montanus
P. ouachitae
P. fourchensis
P. caddoensis
P. metcalfi
P. jordani
P. petraeus
P. kentucki
P. yonahlossee 1
0.49
P. longicrus
P. yonahlossee 2
0.66
P. ventralis
0.63
P. dorsalis
P. angusticlavius
P. welleri
0.57
Mitochondrial data only
P. wehrlei
P. punctatus
P. websteri
P. nettingi
P. hubrichti
P. richmondi
P. electromorphus
P. virginia
P. hoffmani
P. cinereus 3
P. cinereus 1
P. cinereus 2
P. shenandoah 2
P. shenandoah 1
P. serratus
0.73
P. vehiculum
P. elongatus
P. vandykei
P. idahoensis
0.1

Figure 1.3: Nuclear *Plethodon* phylogeny. Phylogeny of the salamander genus *Plethodon* based on a combined, partitioned Bayesian analysis of nuclear DNA (nucDNA) only. An asterisk next to a node indicates strong support ($Pp \geq 0.95$). Small open circles on a node indicate $Pp < 0.95$, and these values are listed. The outgroup taxa are excluded (but only from the figure) to facilitate presentation of branch lengths, and the root is indicated with a large open circle.



P. grobmani 2
P. grobmani 1
P. ocmulgee
P. savannah
P. mississippi
0.37
P. glutinosus 1
P. kisatchie
P. sequoyah
P. albagula
P. kiamichi
P. glutinosus 4
P. glutinosus 3
P. kentucki
0.87
P. ouachitae
P. fourchensis
0.39
P. caddoensis
P. yonahlossee 2
P. yonahlossee 1
P. oconaluftee
P. glutinosus 2
0.39
P. cylindraceus
P. chlorobryonis
P. teyahalee
P. aureolus 2
0.79
0.47
P. amplus
P. variolatus
0.34
P. shermani
0.24
P. cheoah
0.60
P. jordani
0.41
P. chattahoochee
P. aureolus 1
Nuclear data only
0.48
P. metcalfi
0.82
P. meridianus
P. montanus
P. petraeus
0.57
P. longicrus
P. ventralis
P. dorsalis
P. welleri
P. angusticlavius
P. wehrlei
P. punctatus
P. websteri
P. virginia
P.hoffmani
0.67
P. nettingi
0.76
P. hubrichti
P. electromorphus
0.70
P. richmondi
P. cinereus 3
P. cinereus 1
P. cinereus 2
P. cinereus 4
P. serratus
0.01
P. shenandoah 2
P. shenandoah 1
P. vehiculum
0.79
P. elongatus
P. vandykei
P. idahoensis

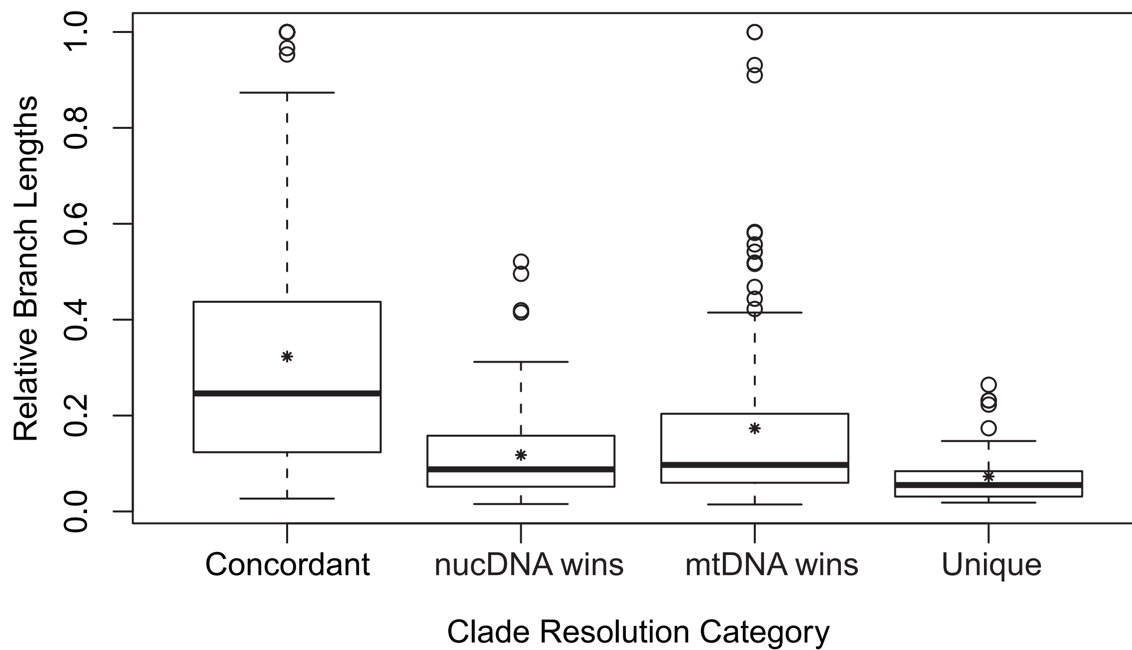


Figure 1.4. Branch lengths by clade resolution category. Box plots of the relative branch lengths for each clade resolution category for the 14 data sets. For each category, the median is indicated by the black bar, and the mean is indicated by the asterisk.

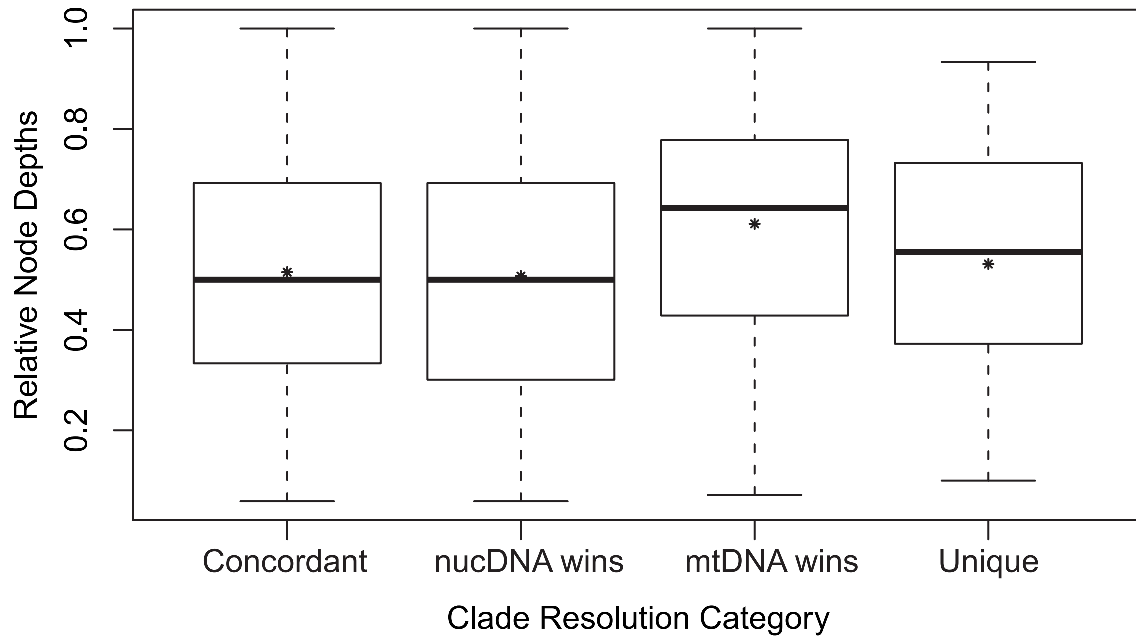


Figure 1.5. Node depths by clade resolution category. Box plots of the relative node depths for each clade resolution category for the 14 data sets. Larger depth indices correspond to shallower nodes. For each category, the median is indicated by the black bar, and the mean is indicated by the asterisk.

Chapter 2

How is rate of climatic-niche evolution related to climatic-niche breadth?

Introduction

The climatic niche is a central concept in ecology and evolutionary biology. It describes the set of temperature and precipitation conditions in which a species can persist (Hutchinson 1957). In general, the climatic niche is important because species are generally limited in the total range of climates they can occupy. For example, few species have physiological tolerances that allow them to occur from arctic regions to the tropics and live year-round in these extreme climates. That is, most species have a limited climatic-niche breadth, which represents the size of the range of climatic values over which a species can persist, on one or more axes of the multivariate climatic niche.

The rate of climatic-niche evolution may also have an important role in many ecological and evolutionary patterns and processes, especially when species have limited climatic niche breadths (reviews in Wiens and Graham 2005; Wiens et al. 2010; Peterson 2011). For instance, climatic-niche conservatism (i.e., a relatively slow rate of change in niche position for populations or species over time), when combined with narrow niche breadths, can be important in limiting geographic range expansion (e.g., Wiens and Graham 2005), which in turn may be important for allopatric speciation (e.g., Kozak and Wiens 2006), patterns of large-scale biogeography and species richness (e.g., Wiens et al. 2006; Rangel et al. 2007; Kozak and Wiens 2010a), determining where invasive species can invade and spread to (e.g., Peterson and Vieglais 2001; Peterson 2003; Thuiller et al. 2005; Mandle et al. 2010), and understanding range shifts and local extinctions of species in response to global climate change (e.g., Tingley et al. 2009). Conversely, rapid niche evolution may allow range expansion and lead to very different patterns.

Previous empirical studies have suggested that the rate of climatic-niche evolution in a clade and the climatic-niche breadths of species in that clade may be related, but implied very different relationships between these variables. Smith and Beaulieu (2009) found that woody plants have slower rates of climatic-niche evolution than herbaceous plants and woody species occupy smaller areas of climate space (suggesting narrower climatic-niche breadths). They

suggested that these narrower climatic-niche breadths are linked to slower rates of climatic-niche evolution, but did not explicitly test the relationship between niche breadth and rate. Kozak and Wiens (2010b) examined the relationship between rate of climatic-niche evolution and diversification rate and found that both rates are faster in the tropics (see also Baselga et al. 2011). In contrast to Smith and Beaulieu (2009), Kozak and Wiens (2010b) suggested that rates of niche evolution are faster in the tropics because climatic niches are narrower in tropical species (e.g., Ghalambor et al. 2006; Kozak and Wiens 2007). However, they did not explicitly test the relationship between niche breadth and rate either.

Why and how should niche breadth and the rate of niche evolution be related? There are many possible relationships between niche breadth and rate. For example, if every species in a region occupies all available climatic zones (i.e., broad climatic niches; Fig. 2.1A), then the potential for divergence between the mean climatic niches of species may be low. Alternatively, if each species occupies a small subset of the climatic zones in the region (i.e., narrow climatic niches; Fig. 2.1B), then the potential for divergence between species' climatic niches should be higher, allowing for a faster rate of niche evolution. Thus, we might expect a relationship between narrow niche breadths and faster rates of climatic-niche evolution (e.g., Kozak and Wiens 2010b). We might also expect a negative relationship between the rate of climatic-niche evolution of a clade and the amount of climatic-niche space that species within the clade occupy relative to the total climatic-niche space occupied by the clade (i.e., the proportional climatic-niche breadth). Nevertheless, there may also be positive relationships between niche breadth and rate (e.g., Smith and Beaulieu 2009). For instance, species with narrow niches might be constrained in their ability to evolve (i.e., a slow rate) due to phenotypic trade-offs during specialization (review in Futuyma and Moreno 1988).

The relationships between niche breadth and rate have not been explicitly tested in either empirical or theoretical studies, but some theoretical studies have addressed related issues. Huey and Kingsolver (1993) addressed the response of species' thermal performance to gradual, constant climate warming. Populations with large performance breadths were predicted to show a greater lag time for their population mean phenotype to catch up to the environmental optimum than populations with narrow performance breadths (eq. 1 in Huey and Kingsolver 1993), implying a negative relationship between niche breadth and rate of niche evolution. Whitlock (1996) demonstrated that specialized species (i.e., narrow niche breadth) may be able to evolve

faster than generalized species (i.e., broad niche breadth) because purifying selection acting on performance in one environment (eliminating genotypes that maximize genotype-environment interactions) is expected to be more effective than selection acting on performance across many environments, allowing specialists to increase mean fitness faster than generalists. This implies that species with narrow niches generally evolve faster than broad niched species in the same environment, but does not directly address if there is a faster rate of changes between different niches.

Variance in climatic-niche breadth among species in a clade and rates of climatic-niche evolution may also be related (Fig. 2.1A, C–D). Species in a clade may have low variance in their climatic-niche breadths if they: (1) have very broad climatic niches (Fig. 2.1A); or (2) have narrow niches near the same position on the niche axis (Fig. 2.1C). Both cases may have low rates of climatic-niche evolution. However, in a clade in which niches are evolving rapidly (Fig. 2.1D), there may be a broad range of niche breadths, especially if niche evolution involves niche expansion of narrow-niched species or niche subdivision of broad-niched species, and if species are at various stages in these processes. Thus, groups with a high rate of niche evolution may have high variance in climatic-niche breadth.

Finally, latitudinal variation in climate may also play an important role in the relationship between climatic-niche breadth and rate of climatic-niche evolution. Previous work suggests that tropical species, in addition to having faster rates of climatic-niche evolution (Kozak and Wiens 2010b), have narrower temperature niches than temperate species (i.e., Janzen's (1967) hypothesis; MacArthur 1972; Vázquez and Stevens 2004; Ghalambor et al. 2006; Kozak and Wiens 2007; McCain 2009; Hua and Wiens 2010). The relationship between lower latitudes and narrower climatic-niche breadths is attributed to greater climatic stability at a given location in the tropics compared to the temperate zone (lower annual variation in temperature; Janzen 1967). Climatic stability is thought to lead to narrower and more distinct climatic zones along elevational gradients, stronger climatic barriers to dispersal, and the evolution of species that occur in relatively narrow climatic zones (e.g., Janzen 1967; Kozak and Wiens 2007; Hua and Wiens 2010). These predictions are partially supported in empirical studies. Kozak and Wiens (2007) found narrower temperature niche breadths for tropical species in a comparison of 30 sister-species pairs of plethodontid salamanders from a wide range of latitudes, while Hua and Wiens (2010) found a similar pattern across 79 sister-species pairs of frogs. McCain (2009)

found that vertebrates have narrower elevational ranges in the tropics (which partially supports the hypothesis of Janzen 1967), but did not examine climatic-niche breadths. Vázquez and Stevens (2004) examined global mean monthly temperature and precipitation ranges and found that temperature does become more temporally stable with decreasing latitude (i.e., narrower range of values as latitude decreases). However, they also found that mean monthly precipitation ranges are larger in the tropics. Thus, Janzen's (1967) hypothesis may only hold for temperature. Here, we explore the relationships between the rate of climatic-niche evolution and climatic-niche breadth in the salamander family Plethodontidae. Plethodontidae contains 419 of 615 recognized salamander species (AmphibiaWeb 2012). Plethodontids occupy a wide range of microhabitats from aquatic to terrestrial, and even arboreal (e.g., Wake and Lynch 1976; Petranka 1998; Blankers et al. 2012). Plethodontidae also contains the only salamander clade that has radiated substantially in the tropics (tropical bolitoglossines; Wake and Lynch 1976; Wiens 2007).

Plethodontids are an excellent study system in which to address the relationship between niche breadth and rate. First, there is a well-resolved, time-calibrated phylogeny for 250 plethodontid species, along with extensive climatic data for each species (Kozak and Wiens 2010b). These climatic data can be used to estimate the niche breadth of each species, and the phylogeny allows estimation of rates of niche evolution within clades. Second, previous work (Kozak and Wiens 2010b) showed extensive variation between clades in their rates of climatic-niche evolution (for which rates were estimated based on changes in mean multivarite niche position among species). Third, there is substantial variation in niche breadths among species (Kozak and Wiens 2007). However, the relationship between breadth and rate has only been implied (Kozak and Wiens 2010b), and has not been explicitly tested in plethodontids (or other groups).

Materials and Methods

Phylogeny

We used the same time-calibrated phylogeny used by Kozak and Wiens (2010b). This phylogeny (from Adams et al. 2009) contains 250 putative plethodontid species, and was constructed by combining the trees from Kozak et al. (2009; for higher-level relationships and

North American species; from multiple nuclear and mitochondrial genes) and Wiens et al. (2007; for tropical bolitoglossines; from mitochondrial genes). Although not every species has data for every gene in these data sets, focused analyses suggest that missing data are not generally problematic for either one, a hypothesis also supported by extensive results from simulations and empirical data (for results and review see Wiens and Morrill 2011). This phylogeny is generally strongly supported and similar to other recent estimates with more limited taxon sampling (e.g., Vieites et al. 2011). The phylogeny is time calibrated using three potential root ages for the family (48, 61 and 69 Million years ago, Myr; from Wiens 2007). These three potential root ages influence both the absolute and relative ages of clades, although we find that they generally have little impact on our results. These root ages are somewhat younger than other estimates (e.g., those based on mitochondrial and nuclear data; Roelants et al. 2007; Vieites et al. 2007, 2011), but the important issue for our study is whether the ages of more nested clades (e.g., genera) are likely to be accurate. Recent analyses by Zheng et al. (2011) using multiple nuclear genes across all salamanders (using Bayesian estimation in BEAST; Drummond et al. 2006; Drummond and Rambaut 2007) show that their preferred results are very similar to those of Kozak et al. (2009; their Fig. 4) for the median root age (61 Myr) for more nested clades. Specifically, Zheng et al. (2011) estimate the crown-group age of Spelerpinae at ~40 Myr, *Desmognathus-Phaeognathus* ~40 Myr, *Aneides* ~30 Myr, and *Plethodon* ~45 Myr, whereas Kozak et al. (2009) estimate Spelerpinae ~36 Myr, *Desmognathus-Phaeognathus* ~36 Myr, *Aneides* ~30 Myr, and *Plethodon* of ~42 Myr (i.e., estimates are within ~5 Myr).

We used the same 16 clades as Kozak and Wiens (2010b) for comparative analyses (Fig. 2.2; see below). These 16 clades were chosen for several reasons. First, because they correspond to previously recognized groups (e.g., genera), it is possible to assign many species to these clades even though not all plethodontid species are included in our tree. Second, they are phylogenetically non-overlapping (i.e., no clade is a subset of another), and they together encompass most plethodontid species. Third, each clade contains at least four species that are included in the phylogeny, which facilitates estimation of rates of climatic-niche evolution. Previous analyses (Kozak and Wiens 2010b) suggest that use of alternate clade divisions (given these same constraints) has little impact on the overall patterns of climatic-niche evolution and their rates.

Climatic data and rates of climatic-niche evolution

We used climate data from 16,914 geo-referenced localities from 354 species (mean = 48 locations/species, range = 1–2287 locations/species) for which 19 climatic variables (see Table 1) from the WorldClim data set (Hijmans et al. 2005) had previously been extracted (Kozak and Wiens 2010b). Georeferenced localities for each species were carefully vetted to ensure that they correspond to known geographic ranges, and that the broad-scale geographic distribution of wide-ranging species was represented. Not all 354 species belong to the 16 focal clades (e.g., the genus *Thorius*), and these were dropped from subsequent clade-level analyses of climatic-niche breadth for a total of 299 species (see below). We performed a principal components analysis (PCA) on the correlation matrix of these 19 climatic variables. PC1–PC5 account for >90% of climatic variation among species. We used PC1–PC4 (explaining a total of 89.22% of the variation) in all subsequent analyses, because these axes each explain more variation than expected by chance (broken-stick distribution; Jackson 1993). Loadings of each climatic variable on each of the first four PC axes are shown in Table 2.1. Of the 12 variables that load strongly onto PC1 (absolute value > 0.5), the four with the largest magnitudes are temperature variables. Of the eight variables that load strongly onto PC2, six are precipitation variables, including the two with the largest magnitudes. The PCA results from Kozak and Wiens (2010b) are similar in magnitude, but often have the opposite sign, presumably due to their use of an additional non-climatic variable (elevation). We obtained a PC score for each locality on each PC axis, and then estimated a mean PC score for each species on each PC axis, by averaging PC scores across localities.

The mean PC score for each species was used as a continuous character to calculate the rate of climatic-niche evolution for each of the 16 clades for each PC axis. Rates of climatic-niche evolution (σ^2) for each PC axis for each clade were estimated using a censored test in the application Brownie v2.1.2 (O'Meara et al. 2006). Brownie yields a maximum-likelihood estimate of the rate of phenotypic evolution based on the observed values of the terminal taxa for a character and the estimated value at the root of the tree, given a tree with branch lengths and a Brownian motion (BM) model of evolution. Kozak and Wiens (2010b) assessed the relative fit of a BM model versus an Ornstein-Uhlenbeck (OU) model of evolution for these 16 clades, and found that BM has the best fit for most clades. Additionally, excluding the four clades which do not consistently fit a BM model (Kozak and Wiens 2010b) does not change the phylogenetic

generalized least-squares (PGLS; Martins and Hansen 1997) results for the multivariate analysis of climatic niche breadth in this study (see below; results not shown). A multivariate estimate of rate for each clade was calculated using an unweighted average of the rate estimates for the individual PC axes. Calculating a multivariate rate using a weighted average of the PC axes (each PC axis is weighted by the relative amount of variation it accounts for) does not change the overall results (see Online Supplement B.3 for PGLS results). Hereafter, we use the term "multivariate" to refer to the unweighted average of rates on the PC axes (or niche breadths, see below), because they encompass multiple PC axes simultaneously and to distinguish this metric of rate from those for individual PC axes and climatic variables. We note, however, that this is not a multivariate metric in a strict, statistical sense.

These average estimates of climatic niche across localities for each species might be biased if some parts of the species range were sampled more extensively than others. Estimating rates from the midpoint of the range of each climatic variable may be less sensitive to this bias than use of the mean (although this approach may instead be more sensitive to outliers). Spearman rank correlation of the midpoint PC scores and mean PC scores for each species on each axis are highly correlated (r_s = 0.93–0.98, $P << 0.0001$; for PC1–PC4), strongly suggesting that they should give very similar estimates of rate. Additionally, we expect species to be more common (and thus more likely to be sampled) in parts of their range where climatic conditions are most suitable for them. Thus, if sampling is biased, it should be towards climatic conditions that are optimal for the species.

The estimated rates of climatic-niche evolution do not include all plethodontid species because not all species are included in the phylogeny. However, Kozak and Wiens (2010b) found no significant relationship between the estimated rate for a given clade and the proportion of species sampled within that clade, suggesting that the estimated rates are not strongly biased by incomplete sampling. In addition, they found the highest rates in the most poorly sampled clade, even though poorly sampled clades are more likely to have their rates be underestimated (O'Meara et al. 2006).

In order to calculate climatic-niche breadth for each species on a given PC axis, we subtracted the minimum PC score from the maximum PC score from all localities for that species. A species represented by only one locality has a climatic-niche breadth of zero on all axes. These species are included in our analyses, given that these species presumably do indeed

have very narrow climatic-niche breadths (e.g., additional localities close to the one recorded would likely have nearly identical climate). Niche breadth was calculated separately for PC1–PC4, generating four orthogonal estimates of climatic-niche breadth for each species. As was done for rates, an unweighted average of climatic-niche breadths across PC1–PC4 was calculated for each species, and was used as the multivariate climatic-niche breadth for a given species. Again, using a weighted average of climatic-niche breadths yielded very similar results (see Online Supplement B.3). For each of the 16 clades, the mean and variance in climatic-niche breadth of all species within the clade were calculated for each PC and for the multivariate climatic-niche breadth. These estimates of climatic-niche breadth included all species in a clade for which we had climatic data (total among 16 clades: 299 species), even if that species was not represented in the phylogeny. Because the 16 clades represent well-supported, well-established taxonomic groups (e.g., genera), we were able to unambiguously assign species not present in the phylogeny to a clade when calculating mean climatic-niche breadths for species within the clade, rather than restricting these estimates to only the species in the tree. Further, our estimates of climatic-niche breadth (both mean and variance) for each clade are strongly correlated between these two sets of taxa (all species in phylogeny vs. all species with climatic data; for PC1–PC4: r_s = 0.79–0.99, all $P < 0.0005$).

Finally, the mean proportional climatic-niche breadth for each clade was calculated. This value represents the extent to which species in a clade occupy the full range of climatic conditions that the clade occupies (on a given climatic-niche axis). First, a climatic-niche breadth for each clade was determined by subtracting the minimum value for each PC score from the maximum value for all localities for all species contained in the clade. Next, the estimates of climatic-niche breadth for individual species (see above) were divided by this clade-level niche breadth to get a proportional niche breadth for each species (i.e., the proportion of the clade's niche breadth that a given species occupies). Proportional niche breadths were then averaged across all species within a clade to estimate a mean proportional climatic-niche breadth for each clade.

The estimates described above were calculated for each root age, for each PC axis, and for several individual climatic variables: BIO1 (annual mean temperature), BIO4 (temperature seasonality), BIO5 (maximum temperature of the warmest month), BIO6 (minimum temperature of the coldest month), BIO7 (temperature annual range), BIO12 (annual precipitation), and

BIO15 (precipitation seasonality). Plethodontid salamanders are thought to be sensitive to temperature (BIO1), particularly to extremes of temperature (BIO5, BIO6), and to be dependent on mesic environments, which require higher precipitation (BIO12; e.g., Petranka 1998; Kozak and Wiens 2010a). BIO4, BIO7, and BIO15 reflect climate seasonality, and allow us to more directly address predictions made from Janzen's hypothesis, which is based on latitudinal differences in annual temperature variability (see also Vázquez and Stevens (2004) for precipitation). These last three variables could be considered measures of climatic-niche breadth on their own. However, by calculating our metric of climatic-niche breadth with these variables, we are looking at how large or small the range of temperature and precipitation seasonality is across the range of each species within a clade. We found that results were generally similar across the different root ages. The results presented below are those for the 61 Myr root age (i.e., the intermediate value between 48 and 69 Myr).

Comparative Phylogenetic Analyses

In order to explore the relationship between the rate of climatic-niche evolution and climatic-niche breadth among clades, we performed analyses using PGLS (Martins and Hansen 1997), implemented in the R-package CAIC (Orme 2007) with R version 2.11.1 (R Development Core Team 2010). The 250-species tree was pruned so that each clade was represented by a single species (given the time-calibrated tree, all species in a clade have the same time-span to the root of the clade, making the choice of species irrelevant). This 16-clade tree was then used for PGLS (Fig. 2.2). Based on the predictions described in the introduction, we tested if a clade's multivariate rate of climatic-niche evolution was: (i) negatively related to the mean multivariate climatic-niche breadth among the species in the clade, (ii) positively related to the variance in multivariate climatic-niche breadth among species in the clade, or (iii) negatively related to the mean proportional multivariate climatic-niche breadth of a clade. We then tested the same three relationships for each of PCs 1–4 separately, and for each of the individual climatic variables (BIO1, BIO4–7, BIO12, and BIO15).

In order to test the hypothesis that climatic-niche breadths are narrower in the tropics, we used PGLS with the 250-species phylogeny to test if the multivariate climatic-niche breadth for a given species is positively related to the mean absolute value of latitude of the localities where that species occurs. We expected narrower climatic-niches as the absolute value of latitude

approached zero. We also tested for a relationship between latitude and niche breadth for individual climatic variables (BIO1, BIO4–7, BIO12, and BIO15). Alternatively, we treated species as being either temperate or tropical, and tested for a difference between the mean values for each group using non-phylogenetic methods (i.e., assuming that latitudinal differences within tropical and temperate regions are unimportant and might obscure this relationship). A Wilcoxon rank-sum two sample test (non-parametric *t*-test) was used to test for differences between mean niche breadths of tropical versus temperate species (given that climatic-niche breadths are not normally distributed). There is a distinct separation in latitudinal distributions between tropical species (below 25°N; only two otherwise tropical species are found just north of the Tropic of Cancer) and temperate species (above 25°N). This break was used to categorize species, and agrees with previous classifications of clades as temperate or tropical (e.g., Kozak and Wiens 2010b).

Janzen's (1967) hypothesis suggests that tropical niche breadths should be narrower primarily due to limited temperature seasonality in the tropics. Subsequent research has found that tropical species also have faster rates of niche evolution (Kozak and Wiens 2010b), which suggests the following scenario: limited seasonality leads to narrower niche breadths which lead to faster rates of niche evolution. Each of the analyses above focuses on the second part of this scenario: the relationship between niche breadth and rate. Since there are many possible relationships between niche breadth and rate, we also tested for a relationship between seasonality and rate of niche evolution. Based on the scenario described above, we might expect to find temperature seasonality negatively related to rates of temperature niche evolution. We may also expect this relationship for precipitation seasonality and rate, although the latitudinal pattern will be reversed (precipitation has greater seasonality in the tropics; Vázquez and Stevens 2004). For each species, we calculated a mean BIO4 (temperature seasonality) and mean BIO15 (precipitation seasonality) value. These values were then used to calculate mean temperature and precipitation seasonality values for each clade (mean of species means). We then used PGLS to test for relationships between (i) mean temperature seasonality vs. rate of climatic-niche evolution for BIO1, BIO5 and BIO6 (the basic temperature variables; Table 2.1) and (ii) mean precipitation seasonality vs. rate of climatic-niche evolution for BIO12 (annual mean precipiation).

Finally, the analyses described above involve many tests and *P*-values. Although a Bonferroni correction is often appropriate (e.g., Rice 1989), the application of such a correction to every result in the study would be highly problematic (e.g., many analyses are repeated to address the robustness of the results to different clade ages). Therefore, we did not apply such a correction to every *P*-value in the study, but we do not discuss results with $P > 0.01$. Furthermore, our major result is that the relationship between niche breadth and rate is either absent or positive, and so our results do not support a relationship between narrower breadths and faster rates, regardless of whether or not there is a Bonferroni correction.

Results

For each clade, estimates of the multivariate rate of climatic-niche evolution, the multivariate mean and variance in climatic-niche breadth, and the mean proportional climatic-niche breadth are summarized in Table 2.2. Estimates for each individual PC and the individual climatic variables are summarized in Online Supplements B.1 and B.2, respectively. Results of all PGLS analyses for each root age are reported in Online Supplement B.3.

There is no significant relationship between the mean climatic-niche breadth of species in a clade and the clade's rate of climatic-niche evolution, in either the multivariate analysis ($r^2 = 0.002$, $P = 0.864$) or for individual PCs ($r^2 = 0.027$–0.195, $P \geq 0.087$). Similarly, the relationship between variance in multivariate climatic-niche breadth of a clade and the multivariate rate of climatic-niche evolution is also not significant ($r^2 = 0.177$, $P = 0.105$). The variance in climatic-niche breadth for PC2 is positively related to the rate of climatic-niche evolution for PC2 ($r^2 = 0.302$, $P = 0.027$), but is not significant ($P < 0.01$) for any root age (see Online Supplement B.3). PC1, PC3, and PC4 have no significant relationship between variance in climatic-niche breadth and rate of climatic-niche evolution ($r^2 = 0.149$–0.256, $P \geq 0.046$). These results are robust across all three root ages (see Online Supplement B.3).

The rate of climatic-niche evolution for both temperature seasonality (BIO4) and annual precipitation (BIO12) is positively related to both mean climatic-niche breadth (BIO4: $r^2 = 0.324$, $P = 0.021$, Fig. 2.3A; BIO12: $r^2 = 0.440$, $P = 0.005$, Fig. 2.3C) and variance in climatic-niche breadth for these variables (BIO4: $r^2 = 0.442$, $P = 0.005$, Fig. 2.3B; BIO12: $r^2 = 0.544$, $P = 0.001$, Fig. 2.3D). There are no significant relationships between rates of climatic-niche

evolution and climatic-niche breadth (mean or variance) for the remaining individual climatic variables (BIO1, BIO5–BIO7, BIO15; see Online Supplement B.3). Mean proportional climatic-niche breadth is not significantly related to the rate of climatic-niche evolution for the multivariate analysis ($r^2 = 0.060$, $P = 0.359$), nor for any of the single variable analyses across all root ages (see PGLS results in Online Supplement B.3).

Multivariate climatic niches of tropical species are generally similar in width to those of temperate species. PGLS analysis was not significant for any root age (Fig. 2.4; Online Supplement B.3). One temperate species, *Ensatina eschscholtzii*, is an outlier, with a very wide multivariate climatic-niche breadth (8.89; Fig. 2.4). Nevertheless, the ranges of climatic-niche breadths are very similar for temperate species (range: 0.00–8.89; mean = 2.18; without outlier range: 0.00–7.25; mean = 2.13) and tropical species (range: 0.00–7.26; mean = 2.31). A Wilcoxon rank-sum two-sample test shows that mean multivariate climatic-niche breadth for temperate species is not significantly different from that for tropical species ($W = 7989.5$, $P = 0.676$; includes outlier; Online Supplement B.4).

Similar to the multivariate results, the climatic-niche breadths based on single climatic variables (Online Supplement B.2) either show no relationship with latitude (BIO1, BIO5, BIO12, BIO15), or a very weak positive relationship with latitude (BIO4, BIO6–7: $r^2 = 0.023$–0.062, $P \leq 0.016$; Online Supplement B.3). These results are generally similar across different root ages (Online Supplement B.3). Wilcoxon tests for differences in mean tropical climatic-niche breadth versus mean temperate climatic-niche breadth are significant for BIO4 and BIO7 ($P << 0.001$; Online Supplement B.4), with mean tropical niches being narrower than mean temperate niches for those two variables. However, results from Wilcoxon tests are not significant for BIO1, BIO5–6, BIO12, or BIO15 ($P = 0.151$–0.742; Online Supplement B.4).

The rate of climatic-niche evolution for BIO5 (maximum temperature of the warmest month) is significantly negatively related to the mean temperature seasonality for each clade ($r^2 = 0.409$, $P = 0.008$; Fig. 2.5). Interestingly, there is also an obvious geographic split, as seen in Figure 2.5. The seven tropical clades have the greatest range of rates for BIO5, and the most limited temperature seasonality. The three clades found primarily in western North America have intermediate temperature seasonality and rates for BIO5. The remaining six clades, with the slowest rates for BIO5 and greatest temperature seasonality, are found in eastern North America. The relationship between temperature seasonality and rate of climatic niche evolution

for BIO1 and BIO6 was also negative, but only significant for BIO1 for the 61 Myr root age (see Online Supplement B.3 for PGLS results). There was no significant relationship between precipitation seasonality and rate of evolution for BIO12.

Discussion

The rate of climatic-niche evolution is important to many topics, from responses to global climate change, to speciation, to large-scale patterns of biodiversity. It is often assumed that climatic-niche breadth and the rate of climatic-niche evolution are closely related (e.g., Smith and Beaulieu 2009; Kozak and Wiens 2010b). However, the relationship between these variables has not been explicitly tested and the expected relationship between them is uncertain.

We found that rate of climatic-niche evolution and mean climatic-niche breadths are generally unrelated among the 16 clades of plethodontid salamanders. For PC-based analyses, only results for PC2 approach significance. Kozak and Wiens (2010b) found that PC1 largely accounts for the climatic variation between tropical and temperate species (see Fig. 1b in Kozak and Wiens 2010b), whereas PC2 largely accounts for within-region variation in climate among species. In both studies, climatic variables have similar loadings on these axes, with PC1 having stronger loadings for temperature variables than precipitation variables, and PC2 having stronger loadings for precipitation than temperature variables (but these axes are not solely temperature vs. precipitation; Table 2.1). Our results suggest that variance in climatic-niche breadth on PC2 is positively related to rates of climatic-niche evolution, suggesting that precipitation may be important in explaining within-region variation in the climatic-niches of plethodontid salamanders, although this result was not significant.

For temperature seasonality (BIO4) and annual precipitation (BIO12), we find a strong positive relationship between the rate of climatic-niche evolution and both mean climatic-niche breadth and variance in climatic-niche breadth, even though previous analyses of plethodontids (Kozak and Wiens 2007, 2010b) predicted a negative relationship between mean climatic-niche breadth and rate. In contrast, the remaining temperature variables (BIO1, BIO5–7) and precipitation seasonality (BIO15) show no relationship between niche breadth and rate. Finding the same pattern in both a temperature and precipitation variable suggests that the positive relationship is consistent across the two major climatic axes.

Why should rates of niche evolution be faster for clades in which species have broader climatic-niches? This may depend on the climatic variable. For precipitation, the seven clades with the fastest rates of precipitation niche evolution and the broadest, most variable precipitation niches (Fig. 2.3C–D) all inhabit the very wettest environments (the tropics and the Pacific northwest of North America), for which the distinction between the available moisture regimes is not wet versus dry, but rather wet versus very wet. This dichotomy is reflected in the maximum mean precipitation values among species of these seven clades (~2500–4800 mm for the seven fastest clades vs. 1400–2150 mm in the nine slower-evolving clades on this niche axis). Also, these seven clades have the greatest ranges of mean precipitation values among species within the clade (range among species of ~1900–3600 mm for the seven fast clades vs. ~260–1550 mm for the nine slow clades). This pattern suggests that these seven faster-evolving clades live in generally wetter environments, with a much wider range of precipitation regimes compared to the nine slower-evolving clades. It could be that in these seven faster-evolving clades, the basic moisture requirements are generally met, and thus species are able to spread into a variety of wet environments. Likewise, in the nine slower-evolving clades, the species occur in drier environments that may be closer to the limits of their environmental tolerances, constraining both their evolution and the range of environments that individual species can tolerate. This hypothesis should be tested with more in-depth studies of plethodontid environmental tolerances, and with macroevolutionary studies similar to this one in other groups of organisms.

For temperature seasonality, the six plethodontid clades with the slowest rates of temperature seasonality niche evolution and the narrowest, least variable temperature seasonality niches (Fig. 2.3A–B) are all tropical clades. In general, the temperate zone has much greater temperature seasonality than the tropics (Janzen 1967; MacArthur 1972; Vázquez and Stevens 2004). In the tropics, all climatic regimes may have low temperature seasonality, regardless of the absolute difference in mean temperature between them. Thus, given the similarity in seasonality values among species, the rate of evolution is expected to be very slow. Indeed, species in tropical clades show the lowest magnitude and narrowest range of absolute BIO4 values (raw BIO4 values for tropical species range from ~20–400), whereas species in temperate clades show a much higher magnitude and wider range of absolute BIO4 values (raw BIO4 values for eastern North American species: ~500–1200; for western North American species:

~190–950). Interestingly, the three clades with the fastest rates, and broadest, most variable temperature seasonality niches, all occur primarily in western North America, yet have intermediate absolute BIO4 values (but have the widest difference between maximum and minimum raw BIO4 values). This pattern suggests that there is some intermediate level of temperature seasonality that is related to faster rates and broader, more variable niches in plethodontid salamanders, and the very low or very high levels of temperature seasonality constrain evolution along this niche axis, resulting in slower rates.

We also found that the mean proportional climatic-niche breadths of species in a clade were generally not related to the rate of climatic-niche evolution of a clade. However, in the two instances for which the relationship between these two variables approached significance (for PC3 and BIO1), clades that have narrower proportional climatic-niche breadths have faster rates of climatic-niche evolution. BIO1 and other temperature-related variables load strongly onto PC3, and these results are consistent with predictions based on Janzen's (1967) hypothesis.

Contrary to our expectations, we find that multivariate climatic-niches are not strongly narrower in tropical plethodontids relative to temperate species (Fig. 2.4). Interestingly, Janzen's (1967) hypothesis and previous work on niche breadth in plethodontids and frogs (Kozak and Wiens 2007; Hua and Wiens 2010) are both based on temperature, rather than climatic-niches in general. Vázquez and Stevens (2004) suggest that since annual precipitation is more temporally variable in the tropics, temperate precipitation-niche breadths should be narrower than tropical ones. Indeed, we do find that the mean climatic-niche breadth for BIO12 (annual precipitation) for temperate species is somewhat smaller than that for tropical species, but this difference is not significant (temperate mean: 543.01; tropical mean: 990.46; $W = 6934.5$; $P = 0.151$; see Online Supplement B.4 for other variables). Additionally, there is no relationship between rate of climatic-niche evolution and climatic-niche breadth for BIO15 (precipitation seasonality). PGLS analyses of the relationship between latitude and climatic-niche breadth for the remaining individual variables were either non-significant (BIO1, BIO5, BIO12), or weakly positively related (BIO4, BIO6–7), also suggesting that climatic-niche breadth has no strong relationship with latitude.

We did find a significant negative relationship between mean temperature seasonality (BIO4) of species in a clade and the rate of climatic-niche evolution of the maximum temperature of the warmest month (BIO5). This result, and the marginally non-significant

results for the other temperature variables (BIO1, BIO6), suggests that limited temperature seasonality in the tropics is weakly associated with faster rates of temperature-niche evolution, despite the general lack of significant relationships between climatic-niche breadth and rate of climatic-niche evolution and the positive relationship between the rate of evolution of BIO4 (temperature seasonality) and its breadth. There is no relationship between precipitation seasonality and climatic-niche rate for annual precipitation, which suggests that even though the seasonality pattern of precipitation is opposite that of temperature (the tropics have more seasonal precipitation than the temperate zone; MacArthur 1972; Vázquez and Stevens 2004), precipitation seasonality is not related to rates of precipitation niche evolution.

Overall, we make several caveats regarding these analyses and results. First, these analyses address the realized climatic niche of the species involved (i.e., the conditions where species occur, given both abiotic and biotic factors), and not necessarily the fundamental climatic-niche (i.e., the actual physiological tolerances of species to climatic conditions; Hutchinson 1957). Behavioral or plastic responses to climate may also be important in determining climate niche breadths (e.g., Huey et al. 2003). Kozak and Wiens (2010b) showed that rates of climatic-niche evolution are likely influenced by species interactions in plethodontids: the amount of geographic overlap between clades is negatively related to rates of climatic-niche evolution within clades. This suggests that more spatially isolated clades have faster rates of climatic-niche evolution. Their results raise the possibility that climatic-niche breadth may also be influenced by species interactions, especially for the variables (temperature seasonality and annual precipitation) showing a significant relationship between niche breadth and rate. However, PGLS analysis using our climatic-niche breadth data and data on clade overlap (from Table 2 in Kozak and Wiens 2010b) reveals no significant relationships between climatic-niche breadth (for mean, variance, and mean proportional) and clade overlap for all variables across all root ages, including for annual precipitation (results not shown). Furthermore, analyses of individual plethodontid species suggest that climatic variables are generally important in limiting their geographic ranges relative to species interactions (e.g., Kozak and Wiens 2006, 2010a) but this may depend on the particular part of the range limits being considered (e.g., Gifford and Kozak 2012). Thus, our results are not necessarily an artifact of species interactions on climate niche breadths.

Nevertheless, the climatic niche as analyzed here (based on climatic conditions in known localities) may still be only a subset of the fundamental niche, even without the impact of biotic interactions. Peterson et al. (2011) define this as the "existing fundamental niche" based on the idea that climatic tolerances of a species may be underestimated because some climatically distinct (but tolerable) locations are not accessible to individuals of the species due to non-climatic limitations on dispersal (see also Barve et al. 2011). For example, for a species found only on an island, some parts of the mainland may have climatic conditions that do not occur on the island but are nevertheless within the range of tolerances of species on the island, leading to underestimation of the actual climatic tolerances of the island species. A similar situation can be envisioned for species on different continents, or separated by a geographic barrier on the same continent. The existing fundamental niche may be further influenced by changes in climate over time. Thus, the differences we observe in mean climatic-niches between species (used to calculate rate) and climatic-niche breadths may be influenced by non-evolutionary processes like the differences in accessible environments (locally, regionally and temporally) for each individual species (e.g., Godsoe 2010; Soberón and Peterson 2011).

However, it seems unlikely that these differences in accessibility explain our results on climatic-niche breadth and rate of climatic-niche evolution, considering our observations on patterns of proportional climatic-niche breadth. Given that the clades we use tend to each be confined to a single, relatively circumscribed geographic region (e.g., eastern North America, western North America, southern Mexico, lower Central America), the proportional climatic niche breadth addresses the climatic-niche breadth of a species in relation to the total climate space occupied by the clade in the region where they occur. We find that most of the 299 species in our analyses have relatively small multivariate proportional niche breadths (mean: 0.26, range: 0.00–0.94), and most clades have mean proportional climatic niche breadths among species of less than 0.50 (Table 2.2; excepting the *Gyrinophilus* clade, with only 4 species). This pattern suggests that the observed climatic niche distributions and breadths are determined more by the limited tolerances of species to climatic conditions within the regions where they occur, rather than by their inability to reach novel climatic conditions in other geographic regions. In fact, it is hard to imagine why species would be adapted to novel climatic conditions in other regions that they are never exposed to, given that natural selection should favor adaptation to present or recent climates. Finally, it is unclear why there should be strong, non-random relationships

between variables such as niche breadth, niche rate, clade overlap, and species diversification, if the estimated climatic niches of plethodontids are simply artifacts of limited dispersal caused by non-climatic factors.

A second major caveat is that there may be substantial variation in which climatic variables limit geographic ranges, both within clades and within species. For example, within a clade, different variables may be important to different species in limiting their geographic ranges. Further, different parts of the geographic range of a single species could be limited by different climatic variables (e.g., northern vs. southern range limits, upper vs. lower elevational range limits; MacArthur 1972). If there is extensive variation within species and clades as to which climatic variables limit their geographic ranges, it may be difficult to detect patterns at the scale of species and clades. Nevertheless, we do find significant relationships between niche breadth and rate for two biologically important variables (temperature seasonality and annual precipitation).

Third, plethodontids are ectotherms that may be particularly sensitive to the two main axes of climatic variation we examined (temperature and precipitation). Different patterns may occur in other groups, depending on their physiology and other factors.

Fourth, analyses of climatic-niche distribution are potentially influenced by species-level taxonomy. For example, *Ensatina eschscholtzii* may have a wide multivariate climatic-niche breadth because it is "undersplit" by current taxonomy and may actually be multiple species (e.g., the subspecies *croceater, eschscholtzii, klauberi,* and *xanthoptica* are considered potential “genealogical entities” and the subspecies *oregonensis* and *platensis* may represent multiple lineages; p. 992 of Kuchta et al. 2009). In general, temperate plethodontids have been more thoroughly studied for patterns of genetic variation within named species than tropical species, revealing several unnamed lineages that seem to represent distinct species (e.g., in *Desmognathus* and *Eurycea*; Kozak et al. 2005, 2006, 2009). Hence, there may be concern that tropical plethodontids are "undersplit," biasing our estimates of climatic-niche breadth in tropical taxa to be wider on average than more narrow climatic-niche breadths of the more finely split temperate taxa. However, we find no evidence that climatic niche breadths are consistently narrower in either temperate or tropical taxa. Instead, most tropical species are narrowly distributed and therefore tend to be represented by relatively few localities (for 197 species, range: 1–68, mean: 7 localities) compared to temperate species (for 157 species, range: 1–2287,

mean: 98 localities). Thus, tropical species do not have significantly wider niches, and there is little evidence that tropical species are widely distributed and undersplit (or that temperate species are oversplit).

Finally, our analyses involve comparisons among clades, a practice which assumes that there are consistent differences among clades that can be detected. Our results (see also Kozak and Wiens 2010b) show that there are substantive differences in the rate of climatic-niche evolution among clades, and that clades also differ considerably in climatic-niche breadth (Table 2.2). However, it is possible that within-clade variation might swamp between-clade variation, at least in some cases. For example, if many or most clades showed a broad range of within-species niche breadths, from very narrow to very wide, we might not expect any relationship in our between-clade comparisons of within-clade rates of niche evolution and mean species niche breadths. In addition, given only 16 clades, our power to detect weaker relationships between rates of climatic-niche evolution and climatic-niche breadth may be limited.

In summary, we find no strong relationships between the rate of multivariate climatic-niche evolution and the breadth of multivariate climatic-niches in plethodontid salamanders. However, we do find significant positive relationships between rate and breadth for temperature seasonality and annual precipitation. We also find a significant negative relationship between temperature seasonality and rate of climatic-niche evolution for maximum yearly temperature. Overall, our results show that there is not necessarily a tendency for clades of species with relatively narrow niche breadths to have faster rates of niche evolution. Nevertheless, it will be important to test the generality of these results in other clades.

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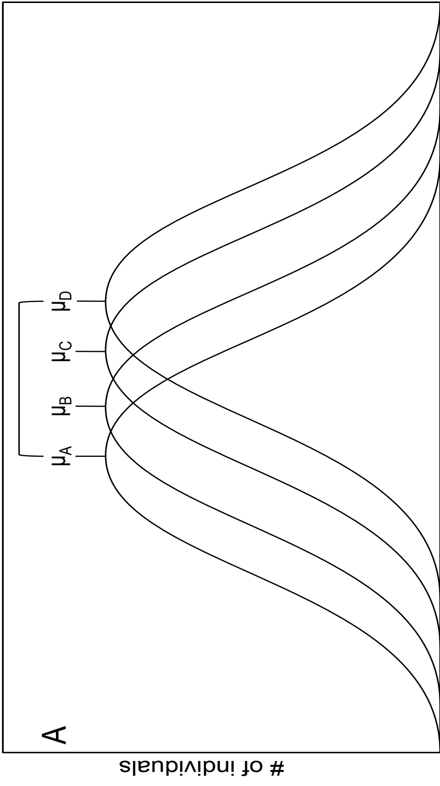
Table 2.1: Factor loadings, eigenvalues, and percent variation explained for the first four axes of the principal components analysis on the 19 climatic variables. PC1–PC4 account for 89.22% of the variation among species, and account for significantly more variation than expected by chance according to a broken-stick distribution.

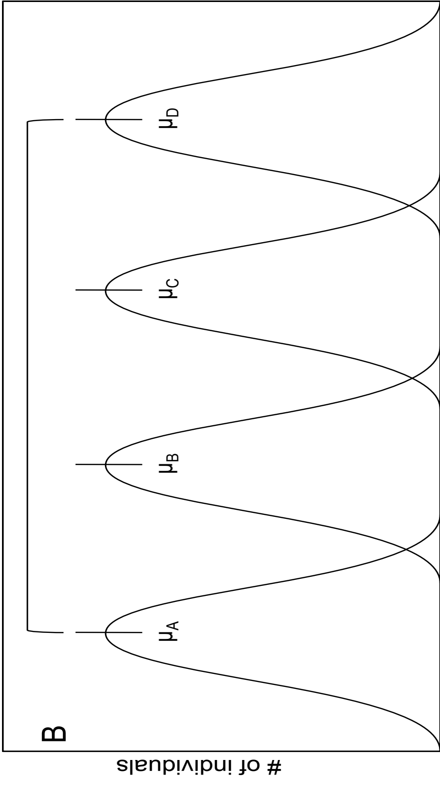
Variable	**PC1**	**PC2**	**PC3**	**PC4**
BIO1 – Annual mean temperature	0.62	-0.24	0.72	-0.08
BIO2 – Mean diurnal range [mean of monthly (maximum temperture – minimum temperature)]	0.12	-0.62	0.08	0.41
BIO3 – Isothermality (BIO2/BIO7 *100)	0.92	0.14	0.01	-0.19
BIO4 – Temperature seasonality (standard deviation *100)	-0.93	-0.20	0.09	0.22
BIO5 – Maximum temperature of warmest month	-0.08	-0.69	0.62	0.33
BIO6 – Minimum temperature of coldest month	0.94	-0.04	0.28	-0.12
BIO7 – Temperature annual range (BIO5 – BIO6)	-0.86	-0.36	0.11	0.29
BIO8 – Mean temperature of the wettest quarter	-0.24	0.01	0.55	-0.64
BIO9 – Mean temperature of the driest quarter	0.74	-0.24	0.10	0.44
BIO10 – Mean temperature of the warmest quarter	-0.21	-0.48	0.81	0.15
BIO11 – Mean temperature of the coldest quarter	0.91	-0.07	0.34	-0.13
BIO12 – Annual precipitation	0.14	0.87	0.32	0.32
BIO13 – Precipitation of wettest month	0.65	0.62	0.18	0.24
BIO14 – Precipitation of driest month	-0.72	0.52	0.29	0.13
BIO15 – Precipitation seasonality (coefficient of variation)	0.88	-0.34	-0.26	-0.01
BIO16 – Precipitation of wettest quarter	0.63	0.63	0.15	0.26
BIO17 – Precipitation of driest quarter	-0.69	0.55	0.29	0.17
BIO18 – Precipitation of warmest quarter	-0.30	0.74	0.45	-0.16
BIO19 – Precipitation of coldest quarter	0.33	0.23	-0.22	0.74
Eigenvalue	8.04	4.22	2.73	1.96
% Variation	42.33	22.21	14.39	10.29

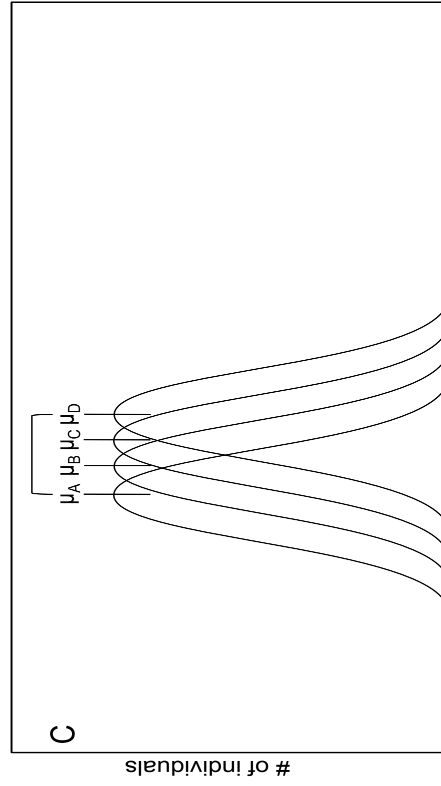
Table 2.2: Summary of data for the 16 plethodontid salamander clades used in the multivariate analyses of climatic niche rate and breadth. N_P refers to the number of species in the clade which are included in the phylogeny and N_C refers to the total number of species in the clade for which we have climatic data (note that all species in the phylogeny have climatic data). Rates and niche breadths are unweighted averages of individual values for PC1–PC4. Estimates from individual PCs and the weighted average are summarized in Online Supplement B.1, and estimates from individual climatic variables are summarized in Online Supplement B.2.

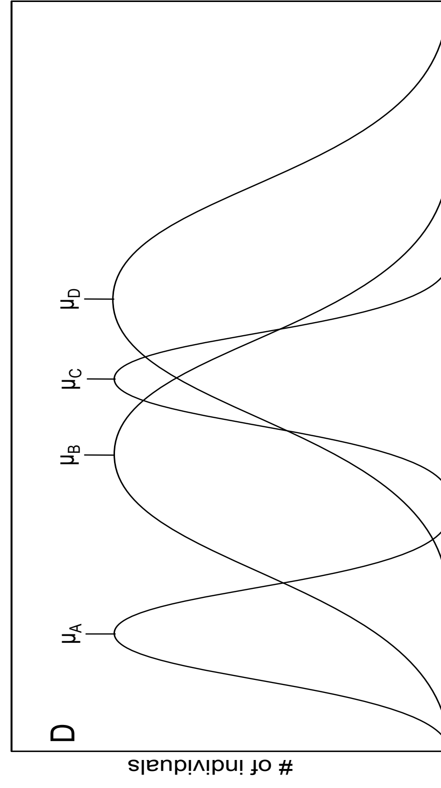
Clade	**N_P**	**N_C**	**Multivariate rate of climatic-niche evolution**	**Mean multivariate climatic-niche breadth**	**Variance in multivariate climatic-niche breadth**	**Mean proportional climatic-niche breadth**
Subgenus *Eladinea* (*Bolitoglossa*)	12	24	0.53	1.95	4.32	0.17
Subgenera *Magnadigitata, Oaxakia, Pachymandra* (*Bolitoglossa*)	20	22	0.26	2.98	6.98	0.31
Subgenera *Bolitoglossa, Mayamandra, Nanotriton* (*Bolitoglossa*)	10	14	0.38	3.86	6.31	0.40
Ixalotriton, Lineatriton, Parvimolge, Pseudoeurycea	37	55	0.22	1.54	2.93	0.18
Chiropterotriton	7	11	0.06	1.84	3.89	0.24
Oedipina	13	16	0.27	1.89	5.17	0.23
Nototriton	6	8	0.12	1.60	5.38	0.24
Batrachoseps	17	26	0.08	2.80	3.26	0.33
Gyrinophilus, Pseudotriton, Stereochilus	4	4	0.03	3.38	3.35	0.62
Eurycea	24	30	0.07	1.30	2.50	0.16
Western *Plethodon*	7	8	0.20	3.03	3.51	0.43
Plethodon cinereus group	9	9	0.04	1.95	1.84	0.37
Plethodon wehrlei-welleri group	7	7	0.05	1.60	0.46	0.37
Plethodon glutinosus group	28	28	0.14	1.53	1.41	0.25
Aneides	5	7	0.08	4.46	7.12	0.49
Desmognathus, Phaeognathus	28	30	0.12	1.65	1.23	0.29

Figure 2.1. Hypothetical examples illustrating different possible distributions of niche breadth and niche divergence among four species in a clade. The X-axis represents a given climatic niche axis (e.g., annual mean temperature, annual precipitation), and the Y-axis represents the number of individuals of a given species that occur at that position on the niche axis (e.g., based on the mean distribution of an individual along that axis). (**A**) A scenario in which all species have relatively wide niche breadths on this niche axis, and limited divergence in their mean values among species (leading to low rates of niche evolution among species). (**B**) A scenario in which all species have narrow niche breadths but extensive divergence in their mean values among species (leading to high rates of niche evolution among species). (**C**) A scenario in which all species have relatively narrow niche breadths on this niche axis, but are clustered around one position along the niche axis, thus showing limited divergence in their mean values among species (leading to low rates of niche evolution among species). (**D**) A scenario in which species have both wide and narrow niche breadths (leading to high variance in niche breadths among species) and intermediate levels of divergence in their mean values among species.


A
# of individuals
Niche Axis
μ_A μ_B μ_C μ_D


B
# of individuals
Niche Axis
μ_A μ_B μ_C μ_D


C
# of individuals
Niche Axis
μ_A μ_B μ_C μ_D


D
# of individuals
Niche Axis
μ_A μ_B μ_C μ_D

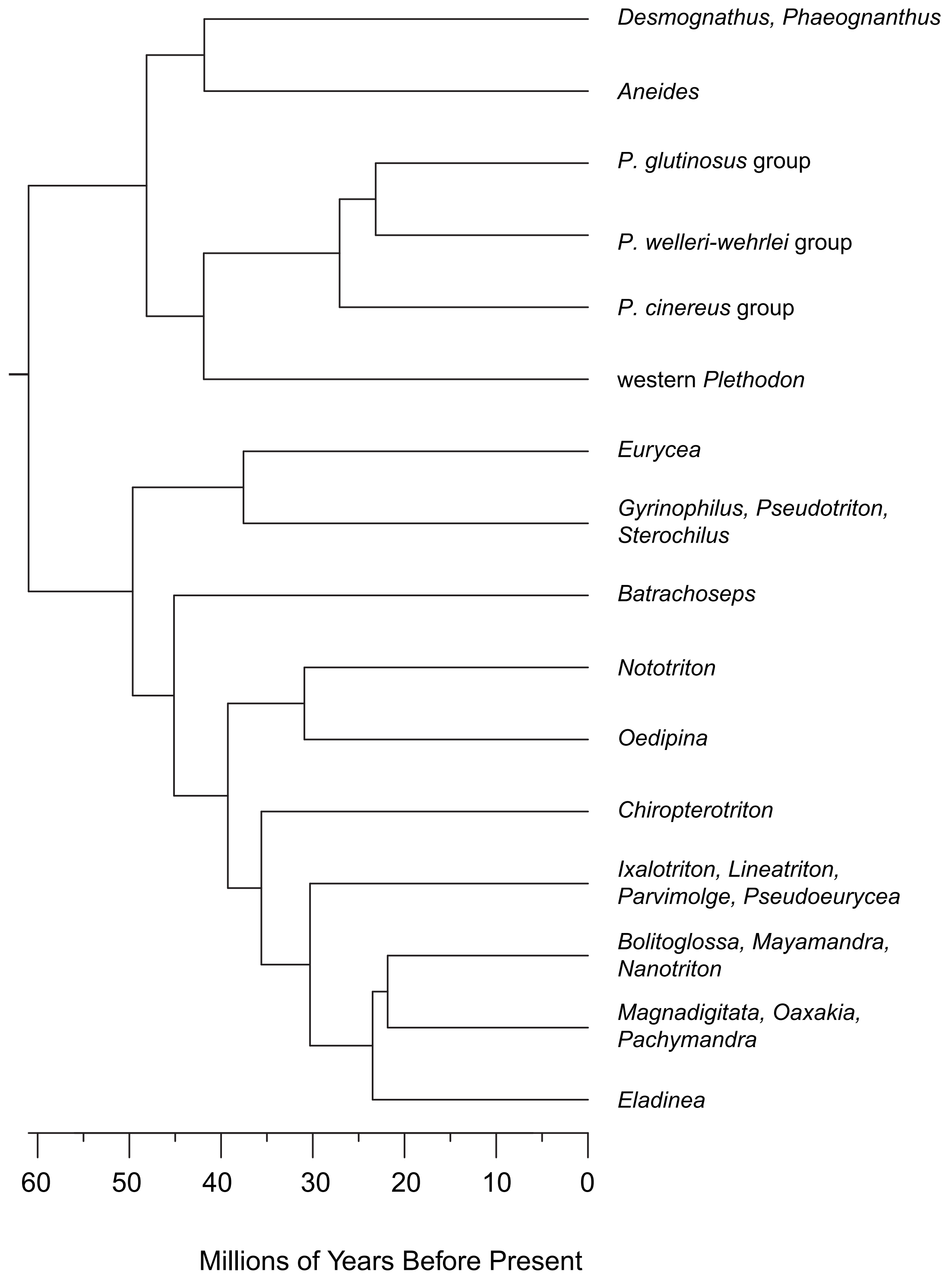
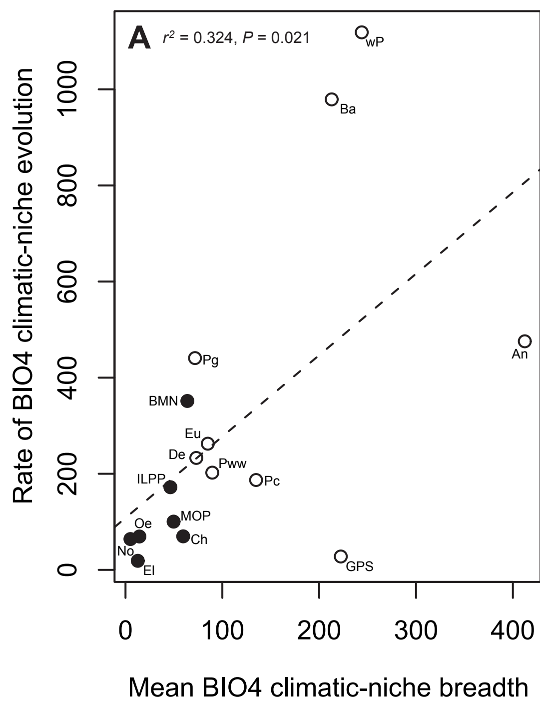
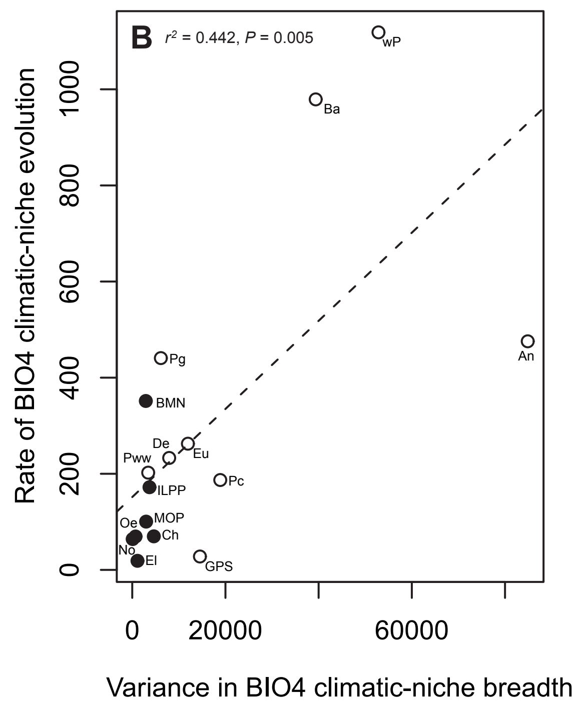
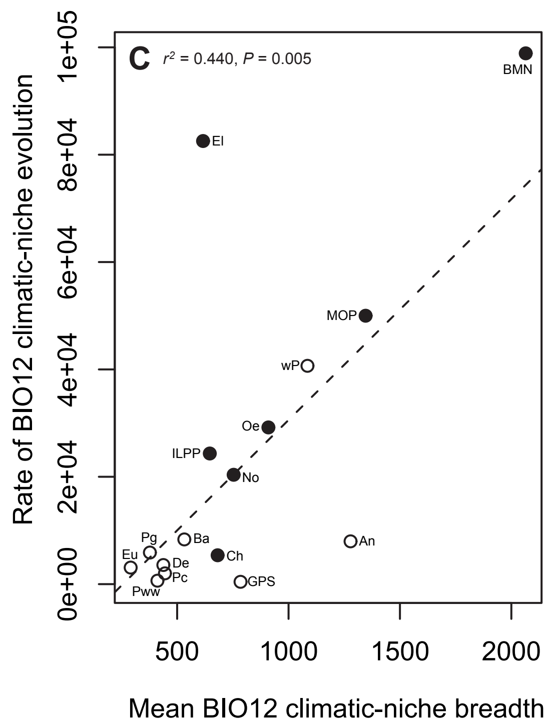


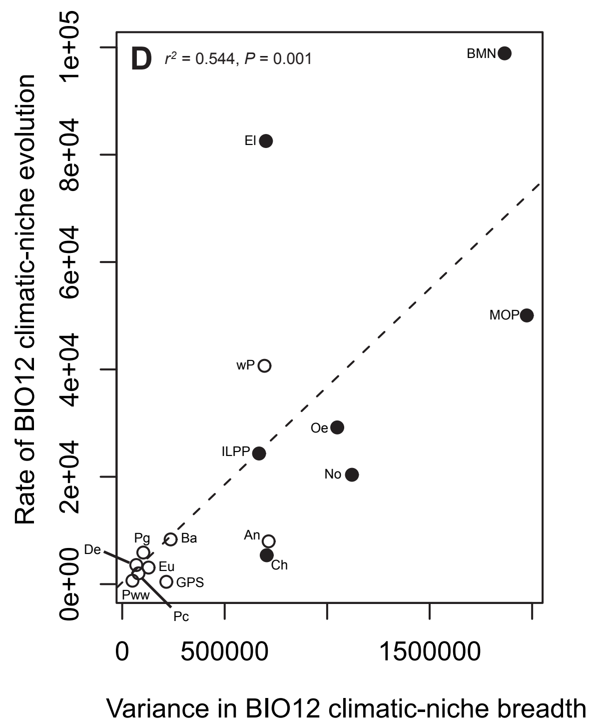
Figure 2.2. Chronogram of 16 major clades of Plethodontidae used in this study (using a root age of 61 Myr).

Figure 2.3. The relationships between the rate of climatic-niche evolution for temperature seasonality (BIO4) and annual precipitation (BIO12), and (A, C, respectively) the mean climatic-niche breadth for BIO4 and BIO12 among species in each clade and (B, D, respectively) the variance in climatic-niche breadth for BIO4 and BIO12. The dashed lines represent PGLS results for the 61 Myr root, and the r^2 and *P*-values are listed in each panel. Filled circles are tropical clades (**BMN** = subgenera *Bolitoglossa, Mayamandra Nanotriton* (genus *Bolitoglossa*); **Ch** = *Chiropterotriton*; **El** = subgenus *Eladinea* (genus *Bolitoglossa*); **ILPP** = *Ixalotriton, Lineatriton, Parvimolge, Pseudoeurycea*; **MOP** = subgenera *Magnadigitata, Oaxakia, Pachymandra* (genus *Bolitoglossa*); **No** = *Nototriton*; **Oe** = *Oedipina*); open circles are temperate clades (**An** = *Aneides*; **Ba** = *Batrachoseps*; **De** = *Desmognathus*; **Eu** = *Eurycea*; **GPS** = *Gyrinophilus, Pseudotriton, Stereochilus*; **Pc** = *Plethodon cinereus* group; **Pg** = *Plethodon glutinosus* group; **Pww** = *Plethodon welleri-wehrlei* group; **wP** = western *Plethodon*). See Online Supplement B.2 for rate, mean, variance, and proportional niche values for each clade, and Online Supplement B.3 for summary statistics from PGLS.


A
r^2 = 0.324, P = 0.021
Rate of BIO4 climatic-niche evolution
Mean BIO4 climatic-niche breadth
wP
Ba
Pg
BMN
Eu
De
Pww
ILPP
Pc
Oe
MOP
No
Ch
El
GPS
An


B
r^2 = 0.442, P = 0.005
Rate of BIO4 climatic-niche evolution
Variance in BIO4 climatic-niche breadth
wP
Ba
Pg
BMN
Pww
De
Eu
ILPP
Pc
Oe
MOP
Ch
No
El
GPS
An


C
r^2 = 0.440, P = 0.005
Rate of BIO12 climatic-niche evolution
Mean BIO12 climatic-niche breadth
BMN
El
MOP
wP
Oe
ILPP
No
Pg
Ba
Eu
De
Pc
Pww
Ch
GPS
An


D
r^2 = 0.544, P = 0.001
Rate of BIO12 climatic-niche evolution
Variance in BIO12 climatic-niche breadth
BMN
El
MOP
wP
Oe
ILPP
No
An
Pg
Ba
De
Eu
Ch
Pww
GPS
Pc

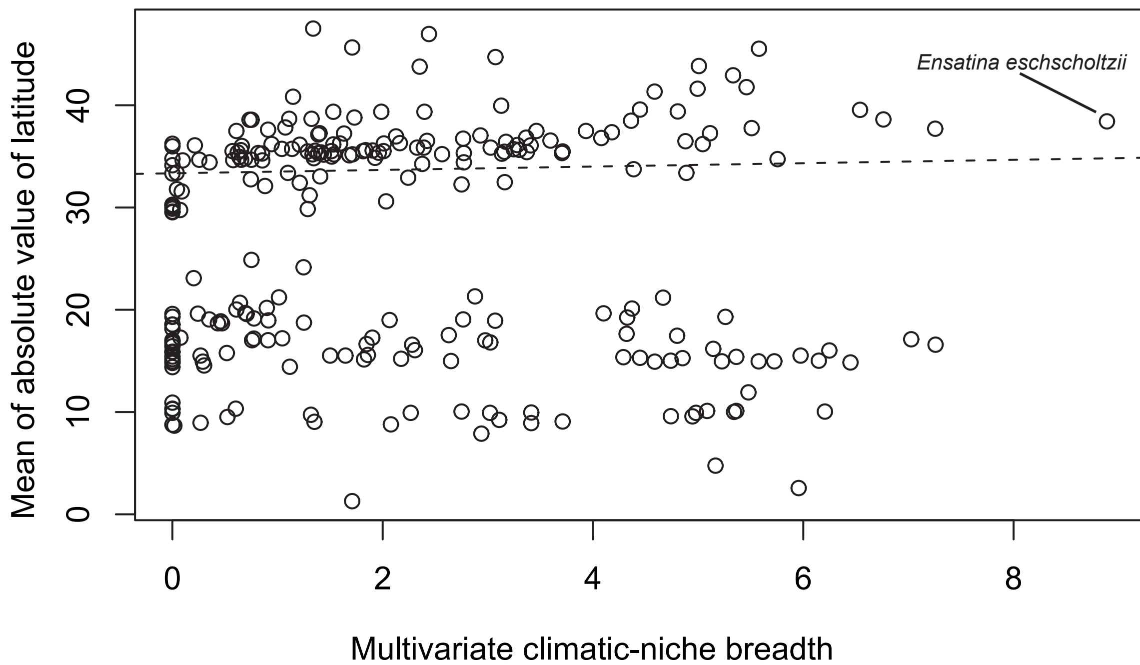


Figure 2.4. Relationships between mean absolute values of latitude and multivariate climatic-niche breadth among 250 species of plethodontid salamanders. Temperate species are represented by the points above 25°, and tropical species are represented by the points below 25°. The dashed line represents PGLS results for the 61 Myr root: $r^2 = 0.018$, $P = 0.032$. See Online Supplement B.3 for summary statistics from PGLS.

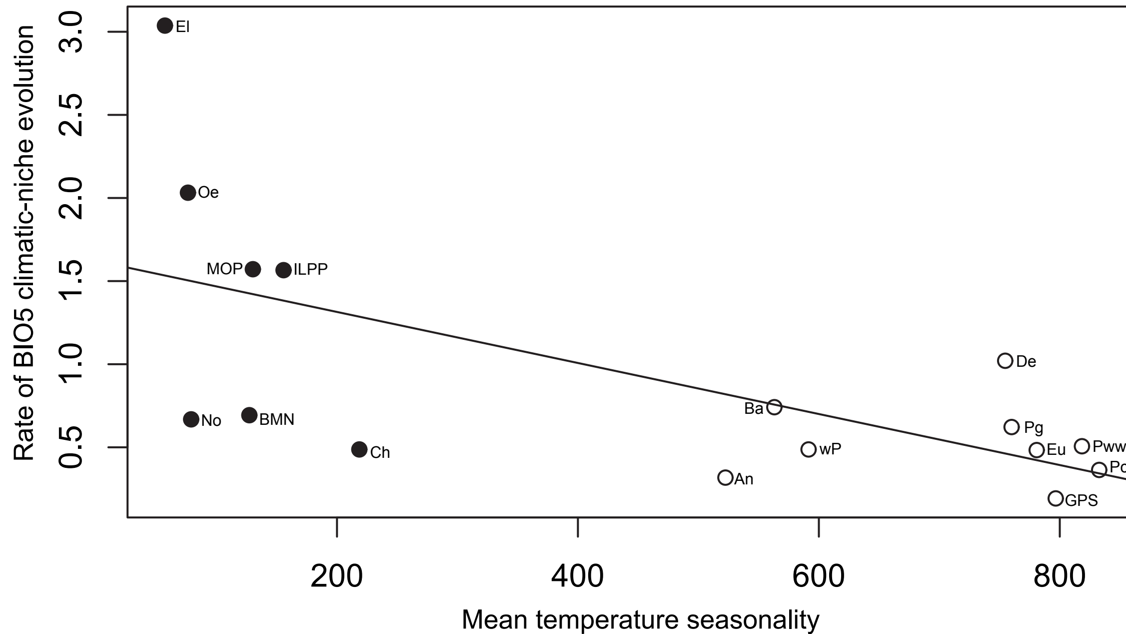


Figure 2.5. The relationships between the rate of climatic-niche evolution for maximum temperature of the warmest month (BIO5) and the mean temperature seasonality of a clade. The solid line represents PGLS results for the 61 Myr root: $r^2 = 0.409$, $P = 0.008$. Filled circles are tropical clades (**BMN** = subgenera *Bolitoglossa, Mayamandra Nanotriton* (genus *Bolitoglossa*); **Ch** = *Chiropterotriton*; **El** = subgenus *Eladinea* (genus *Bolitoglossa*); **ILPP** = *Ixalotriton, Lineatriton, Parvimolge, Pseudoeurycea*; **MOP** = subgenera *Magnadigitata, Oaxakia, Pachymandra* (genus *Bolitoglossa*); **No** = *Nototriton*; **Oe** = *Oedipina*); open circles are temperate clades (**An** = *Aneides*; **Ba** = *Batrachoseps*; **De** = *Desmognathus*; **Eu** = *Eurycea*; **GPS** = *Gyrinophilus, Pseudotriton, Stereochilus*; **Pc** = *Plethodon cinereus* group; **Pg** = *Plethodon glutinosus* group; **Pww** = *Plethodon welleri-wehrlei* group; **wP** = western *Plethodon*). See Online Supplement B.2 for rate and mean seasonality values, and Online Supplement B.3 for summary statistics from PGLS.

Chapter 3

Parapatric divergence of sympatric morphs in a salamander: Incipient speciation on Long Island?

Introduction

Allopatric speciation is widely considered to be the most common geographic mode of speciation (Coyne and Orr 2004). Given this, it is generally assumed that species that are currently allopatrically and parapatrically distributed probably arose through allopatric or parapatric speciation, respectively (but see Losos and Glor 2003) and that species that are partially sympatric likely arose in allopatry as well (Coyne and Orr 2004). There has been some discussion of the possibility that currently allopatric or parapatric species actually originated in sympatry (Coyne 2007; Nosil 2008) and some important theoretical studies support this (e.g., Doebeli and Dieckmann 2000; 2003), but few empirical examples have been produced (e.g., Seehausen et al. 2008; Ingram 2011). Spatial segregation of sympatric ecotypes along an environmental gradient is a key component of the models and examples of this scenario. However, the existing empirical examples involve different habitats at a relatively fine spatial scale (Seehausen et al. 2008; Ingram 2011), and not the macrogeographic scale over which species geographic ranges are usually considered. Here, we address the possibility that sympatric morphs within a species can become parapatrically distributed at a relatively large spatial scale, through a multi-faceted analysis of the Eastern Red-backed Salamander (*Plethodon cinereus*) on Long Island, New York.

Plethodon cinereus is an abundant species ranging from southern Canada to northern North Carolina which typically occurs in two discrete color morphs: the redback or striped morph, and the leadback or unstriped morph (Petranka 1998). The striped morph has a broad orange to red dorsal stripe and dark gray sides, whereas the unstriped morph is uniformly dark gray on the dorsum and sides (Petranka 1998). Hereafter, we use "red" and "lead" for the striped and unstriped morphs, respectively. Color morphology in *P. cinereus* is thought to be polygenic (Highton 1975). There are no documented intermediates between these morphs, and genetic dominance of one morph over another varies by population (Highton 1975). The function of the

red stripe is not entirely clear, but the polymorphism has been implicated in frequency-dependent selection by predators (Fitzpatrick et al. 2009; see discussion therein)

These two morphs are usually found in mixed populations with frequencies of roughly 70% red and 30% lead, but with red morphs found in much higher proportions (90–100%) at higher elevations and the northernmost parts of the species range (Lotter and Scott 1977, Gibbs 1998, Petranka 1998, Gibbs and Karraker 2006; Noël and Lapointe 2010). On Long Island, however, these two color morphs are largely parapatric (Fig. 3.1A), with 100% red populations in the predominantly deciduous forests in the west, and 100% lead populations in the pine barrens habitat in the east (Williams et al. 1968). There are also mixed populations along parts of the north shore of Long Island and down the approximate middle of the island, separating the pure-lead and red populations (Williams et al. 1968).

The red/lead color polymorphism also occurs in other species of *Plethodon*. *Plethodon* contains four well-supported species groups (e.g. Wiens et al. 2006; Kozak et al. 2009; Fisher-Reid and Wiens 2011), and at least eight other species in three of these groups also have sympatric red/lead color morphs (e.g., *P. vehiculum, P. dorsalis, P. serratus;* Petranka 1998). These groups also contain several species that appear to be monomorphic for one of the two morphs (e.g., red: *P. idahoensis*, *P. vandykei*; lead: *P. richmondi*, *P, nettingi*; Petranka 1998). These interspecific patterns raise the possibility that the fixation of color morphs or transitions between morphs may be related to speciation in some *Plethodon*.

These two morphs of *P. cinereus* also show important ecological differences that may be relevant to speciation. On the mainland, these morphs appear to differ consistently on at least two physiological axes. Lead individuals are relatively drought and heat tolerant, whereas red individuals are drought intolerant and cold tolerant (Test 1952, 1955; Williams et al. 1968; Lotter and Scott 1977; Moreno 1989; Gibbs 1998; Gibbs and Karraker 2006; Anthony et al. 2008; but see Petruzzi et al. 2006 for inconsistencies in temperature tolerance). In addition to these physiological differences, diet differences between color morphs and moderate assortative mating by color morph have been documented in mixed mainland populations in Ohio (Anthony et al. 2008).

In this study, we test the hypothesis that the currently parapatric color morphs of *P. cinereus* on Long Island evolved from sympatric morphs that have become spatially segregated along an environmental gradient. We also test if these parapatric populations show ecological,

genetic, and morphological differences suggestive of incipient speciation. From these hypotheses, we make six predictions. First, given a population-level phylogeny based on genetic markers, we predict that Long Island *P. cinereus* populations will form a monophyletic group that is derived from mainland populations where morphs occur in sympatry (i.e., a single population with sympatric morphs invaded Long Island). Similarly, given that patterns of genetic clustering may be more informative than population-level phylogeny within species, we predict that Long Island populations will cluster as separate from the mainland and that pure-red and pure-lead populations on Long Island will also form distinct clusters. Third, we expect to find a gradient in large-scale environmental variables (e.g., annual precipitation, mean temperature) that correlates with changes in morph frequency among populations across the island. Fourth, given the ecophysiological differences previously found between sympatric morphs on the mainland (see above), we expect geographic patterns in microclimate preferences to parallel those for macroclimate. Fifth, we expect that predominantly red and lead populations on Long Island will diverge in other morphological traits besides color (e.g., costal groove number; Williams et al. 1968). Sixth, we expect reduced gene flow between populations having divergent morph frequencies, and that these patterns of gene flow will be related to an environmental gradient between populations but not geographic distance (using a Mantel-test approach; Smouse et al. 1986; Legendre and Legendre 1998).

We find that our results generally support these predictions, and the overall hypotheses of parapatric segregation of sympatric morphs and of incipient speciation on Long Island. We discuss the implications of these results for speciation in this system and others.

Materials and Methods

Study system and sampling

We collected specimens and tissue samples from populations of *P. cinereus* across Long Island (hereafter, LI; N = 61 localities) and adjacent mainland areas in 2003, including Connecticut, New York, New Jersey, Pennsylvania, Delaware, and Virginia (N = 21 mainland localities; for localities and sample sizes see Online Supplement C.1). The GPS coordinates of these localities were used to obtain GIS-based climatic data. Specimens were used for morphological measurements, and liver tissue from these specimens was used to obtain

mitochondrial DNA and microsatellite data. A subset of LI, Connecticut, and New Jersey localities from which specimens were collected in 2003 were selected for microclimate measurements in 2009–2011 (see Online Supplement C.1). This strategy yielded a matched data set of localities for which we had morphological, genetic, microclimatic, and macroclimatic data.

Genetic data

We analyzed patterns of genetic differentiation in *P. cinereus* using both mitochondrial DNA (mtDNA) and microsatellite data. We extracted DNA from alcohol-preserved tissue samples using a Qiagen DNEasy extraction kit. For a complete list of individuals, localities, and type of genetic data collected, see Online Supplement C.1.

One mtDNA locus, ATPase6, was amplified using standard PCR protocols and sequenced using an ABI 3100 automated sequencer (details of primers and PCR conditions in Online Supplement C.2). We initially tested nearly all genes in the mitochondrial genome for a limited sample of individuals (including 12S, 16S, COI, cytochrome b, ND1, ND2, N4) but most showed little variation among LI individuals. Variation in ATPase6 was also low, and so only 187 individuals from 72 localities (53 LI localities, 19 mainland localities) were sequenced. Each locality was represented by 1–6 individuals (mode = 2). Sequence data were edited using Sequence Navigator ver. 1.0.1 (Applied Biosystems), and aligned using MUSCLE ver. 3.8 (Edgar 2004). The alignment was refined manually in Se-Al ver. 2.0a11 Carbon. Sequence data for mtDNA have been deposited on GenBank (accession numbers are in Online Supplement C.1). The following members of the *cinereus* group were used as outgroups: *P. hoffmani, P. hubrichti, P. serratus,* and *P. shenandoah*. Twelve additional *P. cinereus* (5 mainland individuals, 7 LI individuals) and 5 additional outgroups (e.g., *P. richmondi, Ensatina eschscholtzii*) were sequenced for cytochrome b (cytb) to assist in rooting the mtDNA tree (see below; Online Supplement C.1).

Seven microsatellite loci were developed with the bacterial cloning protocol outlined in Glenn and Schable (2005) for a subset of LI *P. cinereus*. We specifically targeted loci that are variable among LI populations. Microsatellites identified by Connors and Cabe (2003) for *P. cinereus* were amplified in a subset of individuals but showed little variation, and therefore were not used. These seven loci were amplified for 233 individuals of *P. cinereus* from 46 localities (31 LI localities, 15 mainland localities). Each locality was represented by 2–7 individuals

(mode = 5). Sampling details are in Online Supplement C.1, and microsatellite loci primers, repeat motifs, and PCR conditions are in Online Supplement C.2. Microsatellite PCR products were visualized using an Applied Biosystems 3730 DNA Analyzer, and chromatograms were initially checked for appropriately-sized fragments using Peak Scanner ver. 1.0 (Applied Biosystems). Raw allele sizes were recorded using Peak Scanner for a subset of the individuals at each locus. Final allele sizes were called and binned automatically using STRand ver. 2.2.3 (Toonen and Hughes 2001). For each locus, the most common raw allele size (identified using Peak Scanner) and the repeat motif number were used to inform STRand in calling fragments. STRand results were manually checked for stutters and refined using the R-package MsatAllele (Alberto 2010).

Finally, each locus was checked for null alleles using Microchecker ver. 2.2.3 (van Oosterhout et al. 2004), and for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) with other loci using GENEPOP on the Web (http://genepop.curtin.edu.au/; Raymond and Rousset 1995; Rousset 2008). There was no significant departure from HWE, and no significant LD between microsatellite loci. Microchecker flagged two loci as potentially having null alleles (evidenced by homozygote excess), which were present at two localities (locus MS2327: LI-Eastport and LI-Moriches) and four localities (locus MS3241: LI-Farmingville, LI-Greenport, Connecticut-Tower Hill, New York-Sleepy Hollow). However, since the homozygote excess was not a significant deviation from HWE, and there was no consistent pattern of null alleles at a given locus across all populations, we retained all loci and localities for subsequent analyses.

Macroclimatic data

Macroclimate data were obtained for each georeferenced sampling locality using ArcGIS 9.3. For each of 75 localities (58 LI, 17 mainland), 19 climatic variables from the WorldClim data set were extracted (Hijmans et al. 2005). These 19 climatic variables represent data on temperature and precipitation (extremes, means, and variability) from weather stations from 1950–2000 (spatially interpolated to localities between weather stations), and have a resolution of 30-arc seconds (~1 km).

Morph frequency data

Data on morph frequencies (i.e., % red individuals) were collected from the same specimens used for molecular and morphometric data. Our categorizations of a locality's morph frequency are generally concordant with those made by Williams et al. (1968). Those authors had larger sample sizes, and sampled LI more broadly (i.e., they have pure-red localities from southwestern LI, in addition to northwestern LI),. The concordance between our two studies suggests that morph frequencies are largely stable over decadal time scales (but see Gibbs & Karraker 2006 for evidence of small, climate-related frequency shifts). For all analyses, we only used localities with morph frequencies based on at least 4 individuals. Of those, sample sizes ranged from 4–25 (mean = 11).

Microclimate data

Fine-scale environmental data (referred to as microclimate data: soil moisture (%); soil pH; ground temperature (ºC); air temperature (ºC); and air humidity (% relative humidity) were collected between March and November during 2009–2011 (see below for details). Each visit began between 13:00–15:00 hours and lasted 1–2 hours. Five, non-overlapping 20 m transects were conducted sequentially in forested areas with a high density of cover objects (e.g., logs, rocks). Cover objects within 2 m of the transect tape were searched for salamanders. When a salamander was found, its color morph and microclimate data were recorded. Instruments used to collect microclimate data included: a Kelway Soil pH and Moisture Meter (for soil moisture and pH; Forestry Suppliers 94302); an Oakton Mini-IR Thermometer (for ground temperature; Forestry Suppliers 89462); and an Oakton Digital Max/Min Thermohygrometer (for air temperature and humidity; Forestry Suppliers 76255). Soil measurements were taken immediately below the location of the salamander when the cover object was removed, and air measurements were taken approximately one meter above this location. In the event that no salamanders were found along the entire transect, these same five abiotic variables were taken once from the center of the transect.

Microclimate data were collected from a subset of the localities previously sampled in 2003. For each sampling date, we randomly selected a locality morph-frequency category (pure-red, pure-lead, mixed), and then a random locality within each category (e.g., one of 17 pure-lead localities). The four mainland localities (two in New Jersey, two in Connecticut) were a separate category. The localities included in this subset were those for which morph frequencies were

well established by previous sampling. *Plethodon cinereus* are most active during the spring and fall, with limited activity in summer (e.g., only after heavy rainfall), and winter (Petranka 1998). Thus, by sampling continuously between March and November, we tried to capture all possible climatic conditions *P. cinereus* experiences in a given active season.

From March to November 2009, two LI localities were visited each week, and one mainland locality per month. For 2010, one LI locality was sampled each week, and two mainland localities were visited per month in the spring (Mar. to May) and fall (Sept. to Nov.), and once per month in summer (Jun. to Aug.). From March to June 2011, two mainland localities were visited per month. Localities lacking salamanders in 2009 were excluded from selection in 2010. Overall, the reduction in LI sampling for the 2010 season was made due to time constraints, whereas the addition of mainland sampling in 2010 and 2011 was made to increase sample sizes.

Morphometric data

Morphometric data were collected from 414 preserved specimens of *P. cinereus* from LI and the mainland (for full list of individuals, localities and raw measurements see Online Supplement C.3). All individuals had a total length (snout-vent-length (SVL) + tail length (TL)) greater than or equal to 60 mm, in order to exclude juveniles (adult total length ~65–125 mm; Petranka 1998). Nine measurements were recorded for each specimen: CG = the total number of costal grooves; SVL = the snout-vent-length; TL = tail length (the posterior end of the cloaca to the tail tip); SG = snout-gular (head) length (the snout to the neck); HW = head width (the lateral side of one eye to the other); AG = axilla-groin (trunk) length (just posterior to the insertion of the forelimb to just anterior to the insertion of the hindlimb); TW = trunk width (just posterior to forelimb insertion); FLL = forelimb length; and HLL = hindlimb length (FLL and HLL are from the posterior insertion of the limb to the tip of the longest digit with limbs outstretched). The latter eight follow Fig. 9 of Good and Wake (1992). Sexual dimorphism appears to be limited in *P. cinereus* and in plethodontid salamanders in general (Petranka 1998) and we therefore did not distinguish males and females.

Data analysis

The following analyses were conducted to address the six major predictions outlined in the introduction. Nonparametric tests were generally used because most data sets violated the assumption of normality (Shapiro-Wilk test).

Microsatellite data

First, to address the monophyly of LI *P. cinereus*, a Fitch-Margoliash tree was constructed using Cavalli-Sforza and Edwards (1967) chord distances (D_C) from the microsatellite data, in Phylip ver. 3.69 (Felsenstein 2004). Populations (i.e., localities) are the terminal units of this phylogeny, and these populations contain allele frequency data from 2–7 individuals (mean = 5). Takezaki and Nei (1996) showed that D_C was the best of seven distances when dealing with highly variable markers like microsatellites. Additionally, Chapuis and Estoup (2007) showed that D_C is less affected by null alleles than Nei's (1972) standard genetic distance (D_S). Robustness of the best tree was evaluated using bootstrapping, with 1000 replicates. Two Virginia localities of *P. cinereus* were used as outgroups, based on likelihood analysis of the mtDNA data, which included species closely related to *P. cinereus* (see below). In order to visualize the geographic origin of populations (LI vs. mainland) and the general morph-frequency category of populations (polymorphic, monomorphic red, monomorphic lead), we reconstructed geographic origin and morph category as two separate characters on the microsatellite phylogeny using maximum likelihood. Ancestral reconstructions were conducted in Mesquite ver. 2.73 (Maddison and Maddison 2006, 2010) using the best tree estimated by the Fitch-Margoliash method. We used the Mk two-rate model for geographic origin and Mk one-rate model for morph frequency category (the most straightforward model for characters with three states). We acknowledge that character reconstruction methods are designed for species and not populations (and not for geographic origins), but these analyses were used to visualize the general patterns implied by the microsatellite data.

We conducted a Bayesian individual-based clustering analysis on the microsatellite data using Structure ver. 2.3.3 (Pritchard et al. 2000; Falush et al. 2003). This analysis provided an additional test of the genetic distinctness of the mainland and LI populations, and the red and lead populations on LI. We used location priors, correlated allele frequencies, and a "no admixture" model of ancestry, for the following reasons. First, our data are composed of relatively few individuals per locality (mode = 5) and markers (seven microsatellite loci), and the

use of location priors can assist Structure in clustering without biasing analyses towards detecting spurious structure (Hubisz et al. 2009; Pritchard et al. 2010). Second, we expect that all populations of *P. cinereus* share some alleles due to common ancestry (in which case correlated allele frequencies should be used; Falush et al. 2003), due to their very large population sizes (Petranka 1998) and an mtDNA phylogeny with little geographic substructuring (Online Supplement C.4). Finally, the "no admixture" model was selected given that most *P. cinereus* on LI currently occur in forest fragments, and are thus currently isolated from each other (i.e., not likely to be experiencing present genetic admixture; e.g., Jordan et al. 2009).

The number of distinct populations (K) supported by the microsatellite data was estimated with Structure by comparing likelihood scores using values of K from 1–15. Each value of K was run for five replicates using a burn-in of 100,000 steps, followed by 2×10^6 steps. Results were analyzed using Structure Harvester web version 0.6.92 (Earl and vonHoldt 2011), which extracts the mean and standard deviation of log likelihood [Ln P(K)] for each K as well as the ΔK metric devised by Evanno et al. (2005; the rate of change of the likelihood function). Both mean Ln P(K) and ΔK were used to determine the best K (Evanno et al. 2005; Pritchard et al. 2010).

Mitochondrial data

A likelihood analysis of the combined mtDNA data (ATPase6, cytb) was conducted using RAxML ver. 7.2.6 (Stamatakis 2006, 2008). The best fitting model of nucleotide evolution was selected for both loci using the Akaike Information Criterion (AIC) in jModelTest ver 0.1.1 (Posada 2008). For ATPase, GTR + Γ was the best model, whereas GTR + Γ + I was selected for cytb. The data were partitioned by gene and codon, and RAxML was run for 200 heuristic maximum-likelihood searches combined with 1000 "fastbootstrap" replicates. We used the GTRGAMMA model for all partitions, as recommended by Stamatakis (2008), given that the GTRGAMMA model uses 25 rate categories when accounting for rate heterogeneity and thus a separate parameter for invariant sites ("I" for cytb) is unnecessary (Stamatakis 2008). Three partition schemes were ranked by AIC values based on the log likelihoods and number of parameters for each scheme. These included (1) no partitions, (2) one partition for each gene (two total), and (3) a partition for each codon position in each gene (six total). Partitions based on both genes and codons had better likelihood and AIC scores than the other two partition

schemes, and only those results are presented here. The mtDNA analysis contained several outgroup species from the *cinereus* group, and *Ensatina eschscholtzii* in order to root the relationships among *P. cinereus* populations and also root the microsatellite tree (given the difficulty of amplifying microsatellite loci across species). The mtDNA tree shows a clade of predominantly southeastern populations as the sister group to other *P. cinereus* populations (Online Supplement C.4). We selected Virginia populations as the root for microsatellite analyses given this result and that the putative sister group to *P. cinereus, P. shenandoah* (Kozak et al. 2009; Fisher-Reid and Wiens 2011; this study) is also endemic to Virginia (Petranka 1998). However, the rooting should have little impact on our conclusions, as long as the root is not within LI.

Macroclimatic data

In order to test for a macroclimatic gradient on LI, we performed a principal component analysis on the correlation matrix of the 19 climatic variables (see Table 3.2) using JMP ver. 9.0.2. Principal components (PC) 1 and 2, which together account for 64.69% of the variation in climate, were plotted to visualize major climatic differences between localities. Principal components 1–3 each accounted for more variation than expected by chance (broken stick; Jackson 1993) and scores for these PCs and select raw variables (BIO1, BIO12) were correlated with morph frequency at each locality using Spearman's rank correlation.

Microclimatic data

We tested for differences in microclimatic occurrences between four types of localities: (i) LI pure-red, (ii) LI pure-lead, (iii) LI mixed, and (iv) mainland. Field data for 26 localities (22 LI, 4 mainland) and 493 salamanders (344 red, 149 lead) were pooled first by locality (all salamanders at a given locality across time), and then by the four groups listed above. While this is not ideal (i.e., we expect some seasonal and annual variation), the sampling was too limited across all time scales at a given locality to remove temporal effects (Fisher-Reid, unpublished). Means of the five microclimate variables (soil moisture, soil pH, ground temperature, air temperature, and air humidity) were estimated for each group. Importantly, these data represent microclimate of individual salamanders. Therefore, occurrence in a particular microclimate can serve as a proxy for individual preferences because salamanders actively select their microhabitat

(Petranka 1998). Each of the five microclimate variables was tested for significant differences between groups for the following comparisons: (i) LI pure-red vs. LI pure-lead (Wilcoxon rank sum two sample test); (ii) LI pure-red vs. LI mixed vs. LI pure-lead (Kruskal-Wallis test); and (iii) LI pure-red vs. LI mixed vs. LI pure-lead vs. mainland (Kruskal-Wallis test). We also used Spearman's rank correlation to estimate the relationship between morph frequency of a locality and each of the five microclimatic variables. For this test, a mean microclimatic value was calculated for each locality for each variable.

Morphological data

We addressed the potential for morphological differences between populations and color morphs by performing two PCAs on the correlation matrix of the morphological data. The first PCA included costal groove number plus the eight morphological variables listed above, while the second did not include costal groove number. Costal groove number is somewhat problematic, since it exhibits a limited range of values among these populations and is therefore not truly continuous. In both PCAs, PC1 was the only PC to account for more variation than expected by chance (broken stick; Jackson 1993). However, as is typical for morphology, PC1 primarily reflects size variation. Thus, we included PC2 in our analyses to include a measure of shape variation. Mean PC scores were estimated for each locality and we used Spearman's rank correlation to test for a relationship between morphology (PC1 and PC2, the latter with and without costal grooves), and color morph frequency.

Spatial analyses

We compared pairwise F_{ST} values in order to test whether or not populations of LI *P. cinereus* were more genetically divergent (i.e., had higher F_{ST} values) than mainland populations over similar geographic distances. Initially, we looked at a subset of mainland localities and the centers of each group of LI localities (LI pure-red, LI pure-lead, LI mixed). The LI groups were compared to seven mainland localities close to LI and arranged roughly in a west-east line (i.e., in Connecticut, New York and New Jersey; six of these seven are pictured in Fig. 3.1A). We estimated pairwise F_{ST} (Weir and Cockerham 1984) values using the seven microsatellite loci and GENEPOP on the Web (Option 6; Raymond and Rousset 1995; Rousset 2008). For this analysis, the geographic midpoint location of LI pure-red, LI mixed and LI pure-lead locality

groups were estimated by taking the average latitude and longitude for each group of localities (see map in Online Supplement C.4). These midpoints were used to estimate the geographic distance in kilometers. Next, we estimated pairwise F_{ST} values and geographic distance between all individual localities and used these values as a distance measure in a partial Mantel test with a group membership matrix (LI pure-red, LI pure-lead, LI mixed, mainland). This analysis tests the hypothesis that group membership predicts pairwise F_{ST} when accounting for geographic distance (i.e., individuals from different localities in the same group have lower a F_{ST} than those in different groups; see description of partial Mantel tests below).

Finally, we conducted a series of single Mantel (1967) tests and partial Mantel tests (Smouse et al. 1986; Legendre and Legendre 1998) to examine if our predictor variables (macroclimate, microclimate) were significantly correlated with our response variables (genetic distance, locality morph frequency) while accounting for any spatial autocorrelation. These analyses included both LI and mainland populations. Specifically, we tested the following five hypotheses: (i) the macroclimate of each locality predicts its morph frequency when accounting for geographic distance between localities (N = 64 localities, all those with morph frequency data); (ii) macroclimate predicts genetic distance when accounting for geographic distance (N = 46 localities, all those with microsatellite data); (iii) microclimate predicts locality morph frequency when accounting for geographic distance (N = 26 localities, all those with microclimate data); and (iv) microclimate predicts genetic distance when accounting for geographic distance (N = 26 localities, all those with both microclimate and genetic data). We also tested the hypothesis that (v) macroclimate predicts microclimate when accounting for geographic distance (N = 26 localities). Because macroclimate and microclimate include several variables of differing scales (e.g., temperature, precipitation), each group of variables was first put through a PCA of the correlation matrix, and the Euclidean distance matrix was then estimated from the PC scores (19 for macroclimate, 5 for microclimate).

Five distance matrices were estimated. The first, "*gen,*" corresponds to genetic distances from the microsatellite data, estimated as chord distances in the program Microsatellite Analyser (MSA ver. 4.05; Dieringer and Schlštterer 2003). The second, "*macro,*" corresponds to a Euclidean distance matrix of the macroclimate data, calculated in R from all 19 macroclimate PC scores ("vegdist" in R package: vegan; Oksanen et al. 2012). The third, "*micro,*" corresponds to a Euclidean distance matrix of the field-collected microclimatic data. Like macroclimate, this

was calculated in R from all five microclimate PC scores. The fourth matrix, "*geo*," is a Euclidean geographic distance matrix and was estimated in kilometers from latitude and longitude coordinates using the "rdist.earth" command in R (package: fields; Furrer et al. 2010), which accounts for the curve of the earth. The fifth matrix, "*morph,*" is based on locality morph frequency (% red), which were first transformed onto an unbounded scale using a logit transformation ("logit" in R package: car; Fox and Weisberg 2011). A Euclidean distance matrix of these transformed proportions was estimated in R using the same method as for the *macro* and *micro* matrices.

Both the single and partial Mantel tests were conducted using the program PASSaGE ver. 2.0 (Rosenberg and Anderson 2011), and significance of the Mantel tests was determined using 10,000 permutations. Because each hypothesis test involved six Mantel tests (three single, three partial), a Bonferroni correction was applied to α for a corrected α of 0.008 (= 0.05/6; Sokal and Rohlf 1995) for each hypothesis.

Results

Phylogenetic analysis of the microsatellite data (Fig. 3.2) shows that LI individuals form a monophyletic group nested within mainland populations, although the bootstrap support is low. Likelihood ancestral reconstructions based on the microsatellite tree also suggests that the ancestor of all Long Island *P. cinereus* was polymorphic and that there was subsequent evolution of pure-lead populations on eastern LI and of pure-red populations on western LI (Fig. 3.2). Pure-lead populations appear to have evolved once on LI (the clade consisting of eastern LI populations) with possible introgression with adjacent mixed populations on the eastern north shore (Cutchogue, Northville). In contrast, pure-red populations appear to have evolved approximately five times on western LI (Fig. 3.2). Some of these pure-red populations may reflect limited sampling (e.g., Brentwood Highway 4; N = 4). However, a group of pure-red populations near Oyster Bay (Old Westbury, Oyster Bay, Woodbury) is supported by very large sample sizes (hundreds of specimens; Williams et al. 1968) and recent field visits.

Structure analyses of the microsatellite data using ΔK selects $K = 2$, whereas log likelihood favors $K = 9$ (Table 3.1). The results with $K = 2$ (Fig. 3.3) divide *P. cinereus* into one exclusively LI cluster and a second cluster consisting of all mainland populations and a few LI

populations (all pure-red). In the K = 9 results (Fig. 3.3), there are five mainland clusters, and four Long Island clusters. The LI clusters correspond to a pure-red cluster, a pure-red + mixed cluster, a mixed + pure-lead cluster, and a pure-lead cluster. These clusters broadly match clades recovered in the phylogenetic analysis (Fig. 3.2). Overall, these results support the genetic distinctness of the mainland and LI populations, and the pure-red and pure-lead populations on LI.

Phylogenetic analysis of the mtDNA data (Online Supplement C.4) includes 101 unique haplotypes. *Plethodon shenandoah* is sister to all *P. cinereus* individuals (see also Wiens et al. 2006; Kozak et al. 2009; Fisher-Reid and Wiens 2011), and a clade of mainland *P. cinereus* individuals from New York, Virginia, Delaware, Maryland and North Carolina is sister to the rest of *P. cinereus*, including all LI populations. Interestingly, 23 individuals from pure-lead localities on LI form a weakly supported clade separate from the rest of the LI individuals. These individuals share a single, non-synonymous nucleotide change in the ATPase6 sequence (from TTT to CTT; Fig. 3.1C). Two additional individuals in this clade are red morphs, one from a mixed locality on the northeastern shore (Wading River), and one from a pure-red locality (Huntington). None of the mainland populations exhibit this mutation.

In the PCA of macroclimatic variables (Fig. 3.4), PC1 separates mainland from LI localities, whereas PC2 separates the LI localities along a climatic gradient that roughly corresponds to morph frequencies (as indicated by the color of the points in the plot). Locality morph frequency was strongly correlated with PC1–3 (PC1: $r_s = 0.588$, $P << 0.0001$; PC2: $r_s = 0.454$, $P < 0.0002$; PC3: $r_s = 0.503$, $P < 0.0001$), but not with raw values of annual mean temperature (BIO1) or annual precipitation (BIO12). The variables which load strongly onto PC1 (BIO6, BIO11, BIO19; Table 3.2) suggest that LI has warmer and wetter winters than the mainland (i.e., coastal insulation; Ricklefs 2008), whereas the variables which load strongly onto PC2 (BIO1, BIO5, BIO10, BIO12, BIO19; Table 3.2) suggest that pure-lead populations experience wetter winters, and overall cooler temperatures compared to pure-red populations. PC3 is dominated by strong loadings from precipitation variables (BIO12, BIO14, BIO15, BIO17; Table 3.2), and suggests that most pure-lead populations experience a drier, more seasonal precipitation regime.

Statistical analyses of the microclimate data (Online Supplement C.5) show that individuals from pure-lead localities on LI are active during significantly colder, drier weather

conditions and in drier and more basic soil than those found at pure-red localities (W = 1927.5–4012; $P \leq 0.00384$ for soil moisture and pH, ground and air temperatures). Interestingly, the temperature pattern is opposite that observed in previous mainland studies, which indicated that leads preferred warmer temperatures (and lower altitudes; see Introduction), whereas the moisture pattern on LI is consistent with mainland studies. Petruzzi et al. (2006) also found divergent temperature tolerances (as measured by maintenance metabolic rates) between morphs at different localities, which suggests that temperature tolerance may be more variable than moisture tolerances. Both microclimatic patterns correspond with the macroclimatic patterns (i.e., higher lead frequencies in cooler and drier localities on LI). All of these differences are non-significant when simply comparing all red individuals on LI to all lead individuals on LI (i.e., including reds and leads from mixed populations; Online Supplement C.5). At mixed localities on LI, lead morph salamanders were encountered on significantly warmer and less humid days compared to red morph salamanders (W = 2736.5–3640; $P < 0.05$), as found in previous mainland studies (see Introduction). A Kruskal-Wallis test comparing pure-red, pure-lead, and mixed localities found mixed localities to be significantly intermediate between pure-red and pure-lead localities for soil moisture and ground temperature (χ^2 = 8.1722-8.8491; $P < 0.05$). A Kruskal-Wallis test comparing the microclimatic data for three LI groups to mainland localities confirms the macroclimatic data: LI salamanders are active under significantly warmer conditions than mainland individuals (χ^2 = 89.3222–97.2697; $P << 0.0001$). The absence of salamanders on some days at both mainland and LI localities appeared to be related to ground and air temperatures: days on which no salamanders were found were significantly warmer than those on which salamanders were found (W = 5505–9725; all $P < 0.006$). Despite these differences among groups of localities (i.e., red, lead, mixed), Spearman's rank correlation of the mean microclimatic data (five raw variables) and locality morph frequency for each individual locality revealed no significant relationships between microclimatic variables and morph frequency (all $P > 0.1$).

Costal grooves show a pattern of variation that closely matches the morph frequencies (Fig. 3.1A,B), with more costal grooves (19 vs. 18) characterizing pure-lead populations in eastern LI, as found by Williams et al. (1968). A PCA of morphology including costal groove number revealed a separation between reds and leads on LI along PC2 (see supplemental figures and loading tables in Online Supplement C.3). Additionally, PC2 including costal groove count

correlated strongly with locality morph frequency (r_s = -0.8065, $P << 0.0001$). In contrast, there was no correlation between morphology and locality morph frequency on PC1 including costal grooves or on PC1–2 not including costal grooves (all $P \geq 0.4375$).

The F_{ST} between LI pure-red and LI pure-lead localities is higher than between any of the mainland localities along a similar west-east gradient, including the two most distant from each other (New Jersey-Dunnfield Creek and Connecticut-Old Lyme; Table 3.3). For example, the centers of LI pure-red localities and LI pure-lead localities are separated by 78.63 km and have an F_{ST} of 0.217. Mainland locality pairs Connecticut-Tower Hill/Connecticut-Monroe, and New York-Orangeburg/Connecticut-Monroe are separated by 79.51 and 79.92 km (respectively) and have an F_{ST} of 0.069 and 0.097 (Table 3.3). A partial Mantel test of the pairwise F_{ST} matrix for all localities against the predictor matrix of group membership (LI pure-red, LI pure-lead, LI mixed, mainland) reveals a significant correlation between F_{ST} and group membership when geographic distance is accounted for (r = 0.4413; P = 0.0001; Table 3.4).

The single and partial Mantel tests (Table 3.4) show that macroclimatic distance has a significant relationship with both genetic distance and locality morph frequency, even when the strong correlation between macroclimate and geographic distance is accounted for. In contrast, microclimatic distance does not predict either genetic distance or locality morph frequency, nor is it related to macroclimatic or geographic distance.

Discussion

In this study, we test the idea that parapatrically distributed organisms can arise through segregation of formerly sympatric ecotypes. Through a multi-faceted analysis of *P. cinereus* salamanders, we show that color morphs that are sympatric on the mainland have become parapatrically distributed on Long Island, and that these parapatric populations have diverged genetically, ecologically, and morphologically. We suggest that *P. cinereus* on LI may represent incipient speciation. Specifically, other parapatrically distributed sets of populations and species may also arise through geographic separation of sympatric ecotypes along an environmental gradient. However, such cases may easily be overlooked if the ecological and morphological differences are subtle (i.e., in our study, the ecotypes have clear differences in color, but different

ecophysiological syndromes within a species or population might often lack such obvious morphological markers).

We acknowledge that the scenario of parapatric differentiation on LI is difficult to prove conclusively. Nevertheless, this scenario is supported both by our genetic data and by other lines of evidence. Under this scenario, *P. cinereus* on LI are derived from an ancestrally polymorphic mainland population. Phylogenetic analysis of our microsatellite data suggests that LI *P. cinereus* are derived from a polymorphic mainland population (Fig. 3.2). Structure analysis of these data also shows that LI *P. cinereus* are genetically distinct from mainland populations (Fig. 3.3). The microsatellite results also support the idea that LI populations diverged genetically after colonizing LI (Figs. 3.2, 3.3).

The scenario of parapatric segregation of sympatric ecotypes is also supported by the well-known geologic history of LI. Long Island was formed by the receding Wisconsin ice sheet relatively recently (25–30 kyr ago), and was likely only accessible to salamanders for a brief period in the last 10–15 kyr (Williams et al. 1968). Thus, *P. cinereus* on LI must have arrived recently from the mainland, and they are currently surrounded by polymorphic mainland populations (Fig. 3.1A; Williams et al. 1968). As the glacier retreated and sea level began to rise, *P. cinereus* is thought to have rapidly expanded its range from southern refugia (Bloom 1983; Petranka 1998). Individuals from polymorphic southern populations on the exposed continental shelf were likely forced to move north by the encroaching Atlantic Ocean and became trapped on LI (Bloom 1983; Lewis and Stone 1991; Stone et al. 2005). Given the intolerance of salamanders to saltwater (Vitt and Caldwell 2009), LI *P. cinereus* are presently isolated from gene flow with mainland populations. Importantly, sea level has never been higher than it is now (Bloom and Stuiver 1963). Thus, the differentiation of populations seen now most likely reflects responses to the current environmental gradient, rather than allopatric differentiation of populations on smaller islands.

The hypothesis of parapatric divergence is further supported by the presence of an environmental gradient across LI along which populations have diverged genetically and morphologically (Fig. 3.4; Table 3.4). Based on the loadings on PC2 and PC3, LI pure-lead populations are found in overall colder and drier environments, that also have warmer and wetter winters. The overall temperature gradient is striking in that mainland leads appear to prefer warmer temperature (e.g., Lotter and Scott 1977; Gibbs and Karraker 2006; Anthony et al.

2008). Within mixed populations on LI, we do confirm that lead morphs are active on warmer, less humid days than red morphs. The between-locality microclimatic data largely confirm the macroclimatic patterns, suggesting that morph-specific physiological tolerances are likely, but more detailed information is needed to confirm this at the single-locality level (see below). Interestingly, our results also suggest that LI has a unique climate and a unique climatic gradient (Fig. 3.4), which may help explain why the pattern of pure-reds and pure-leads in close proximity does not occur on the mainland (but pure-red populations do occur in cooler areas).

We suggest that LI *P. cinereus* may represent a case of incipient speciation, especially for the pure-lead populations on eastern LI. Three lines of evidence supporting this hypothesis: restricted gene flow, local adaptation, and parallels between the within-species pattern in *P. cinereus* and between-species patterns in other *Plethodon.* As described above, pure-lead populations on eastern LI are more genetically different from other LI populations than they are from many more distant mainland localities (Table 3.3). The genetic distinctness of pure-lead eastern LI populations is also supported by phylogenetic and clustering analyses of the microsatellite data (Fig. 3.2, 3.3) and differences in morph frequencies are significantly related to genetic divergence when spatial autocorrelation is accounted for (Table 3.4). The reduction in gene flow between pure-morph populations and its relationship to climatic differences (Table 3.4) meet the basic conditions required by the suite of speciation models based on environmental gradients (e.g., Doebeli and Dieckmann 2000, 2003; Gavrilets 2004). However, even in these genetically distinct pure-lead populations, there is some evidence for introgression with far eastern mixed LI populations (e.g. Baiting Hollow, Cutchogue, and Northville; Figs. 3.2 and 3.3). Clearly, LI *P. cinereus* are not two distinct species yet, however, they do form at least two strongly divergent sets of populations with limited gene flow.

A second line of evidence for incipient speciation on LI is that there is some data suggesting local adaptation to different environments, which could lead to further genetic divergence. Individuals from pure-lead populations tend have more costal grooves (Fig. 3.1B; see also Williams et al. 1968). Costal groove number correlates with vertebral number, which is known to vary in plethodontids and to have a strong genetic component (Jockusch 1997). Jockusch (1997) and others (e.g., Wake 1966) hypothesized that increased vertebral number is an adaptation for a fossorial lifestyle. Eastern LI (the pine barrens, where the pure-lead populations are) is marked by looser, sandier soil, and individuals there may benefit from being better

adapted to burrowing (Williams et al. 1968). Although we found little evidence for divergence in other morphometric variables, plethodontids generally show remarkably similar body shapes across divergent microhabitats (Blankers et al. 2012). In addition to the morphological difference, individuals from pure-lead populations share a non-synonymous change in ATPase6 (Fig. 3.1C), which suggests potential metabolic differences between them and those from pure-red and mixed individuals. The adaptive significance of both traits will need to be investigated in future studies.

The third line of evidence for incipient speciation is the parallel between within-species patterns in LI *P. cinereus* and patterns of between-species divergence among other *Plethodon*. Several other *Plethodon* species are polymorphic for red/lead color morphs, and several others appear to be fixed for one morph or the other (Petranka 1998, Anthony et al. 2008). This suggests that the color polymorphism may be related to speciation, although it is not likely to be driving speciation itself (but see Fitzpatrick et al. 2009 for discussion of color polymorphism function). Based on this study and others (e.g., Lotter and Scott 1977; Gibbs and Karraker 2006; Anthony et al. 2008), color does appear to be linked to climatic preferences in *P. cinereus*. Divergent climatic distributions can drive geographic isolation, adaptation, and genetic divergence between populations (as found here), especially if individuals of one incipient species cannot tolerate the climatic conditions experienced by the other, and many authors have suggested that this general process can lead to speciation (e.g., Moritz et al. 2000; Kozak and Wiens 2007; Sobel et al. 2010). Across plethodontids, rates of species diversification (speciation – extinction) are higher in clades with faster rates of climatic niche evolution (Kozak and Wiens 2010). Interestingly, our preliminary results examining the climatic niches of polymorphic and monomorphic *Plethodon* species suggest that between-species patterns of climatic distributions in fixed red morph and lead morph species do parallel the within-species patterns observed in *P. cinereus*, with pure-red species in cooler, wetter environments (Fisher-Reid and Wiens, unpublished).

We recognize that much additional work is needed on this system. First, as discussed above, the relationship between color morphs, climate, and speciation could be examined across species. The physiological tolerances of both *P. cinereus* morphs and those of other species should also be studied in more detail (e.g., Moreno 1989; Petruzzi et al. 2006). Second, analysis of the LI *P. cinereus* using next generation sequencing could provide clues to loci under

selection, help detect associations of particular alleles with particular habitats, and offer stronger support for the relationships and origins of LI *cinereus* populations. Third, behavioral tests could provide insights on the evolution of prezygotic reproductive isolation. More work is also needed to determine whether the existing patterns of genetic divergence are directly related to different climatic regimes, or possibly to other factors that might be themselves caused by differences in climate (e.g., occurrence in deciduous forests vs. pine barrens). Finally, if there is a speciation-driving environmental gradient on LI, we might expect to find this pattern of parapatric separation in other species that live across the island (Coyne and Orr 2004)

In conclusion, we have here presented evidence for parapatric segregation of formerly sympatric morphs at a large geographic scale in association with an environmental gradient. Pure morph populations are divergent ecologically, morphologically, and genetically, suggesting that this process may eventually lead to parapatric speciation. This pattern of parapatric divergence of sympatric ecotypes along a climatic gradient may represent an early part of the process of parapatric speciation (or range expansion) in many other systems, but may be much harder to detect because the different ecotypes are not as morphologically distinct as they are in *P. cinereus*. This pattern of spatial segregation of pre-existing ecotypes might also allow parapatric speciation to happen more quickly, since the presence of sympatric ecotypes with divergent tolerances may reduce the need to wait for new mutations to allow invasion to a new environment.

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Table 3.1. Results of Bayesian clustering analysis of the microsatellite data using Structure. The best models as selected by log likelihood (first column) and ΔK (second column) are bolded. Bar plot results for these two models are presented in Figure 3.3.

K	Mean Ln P(K)	ΔK
1	-5594.22	NA
2	-5048.26	**65.958**
3	-4730.48	36.008
4	-4596.10	0.107
5	-4459.02	1.461
6	-4357.48	0.848
7	-4273.10	2.245
8	-4204.34	0.438
9	**-4148.66**	49.903
10	-4428.53	1.173
11	-4333.10	0.907
12	-4603.98	0.914
13	-4614.64	0.024
14	-4613.20	0.146
15	-4687.42	NA

Table 3.2. Results from PCA of 19 temperature and precipitation variables from the WorldClim data set from populations of P. cinereus (58 LI, 17 mainland).

Variable	PC1	PC2	PC3
BIO1 – Annual mean temperature	-0.48	0.79	0.07
BIO2 – Mean diurnal range [mean of monthly (maximum temperature – minimum temperature)]	0.90	0.12	-0.01
BIO3 – Isothermality (BIO2/BIO7 x 100)	0.74	-0.14	-0.13
BIO4 – Temperature seasonality (standard deviation x 100)	0.28	0.61	0.34
BIO5 – Maximum temperature of the warmest month	0.22	0.90	0.23
BIO6 – Minimum temperature of the coldest month	-0.86	0.31	-0.06
BIO7 – Temperature annual range (BIO5 – BIO6)	0.78	0.42	0.21
BIO8 – Mean temperature of the wettest quarter	0.70	0.54	0.11
BIO9 – Mean temperature of the driest quarter	-0.59	-0.44	-0.10
BIO10 – Mean temperature of the warmest quarter	-0.30	0.90	0.22
BIO11 – Mean temperature of the coldest quarter	-0.70	0.37	-0.11
BIO12 – Annual precipitation	0.50	-0.62	0.50
BIO13 – Precipitation of the wettest month	0.78	-0.26	-0.05
BIO14 – Precipitation of the driest month	-0.23	-0.45	0.80
BIO15 – Precipitation seasonality	0.50	0.03	-0.80
BIO16 – Precipitation of the wettest quarter	0.81	-0.42	-0.02
BIO17 – Precipitation of the driest quarter	-0.15	-0.18	0.94
BIO18 – Precipitation of the warmest quarter	0.87	0.18	0.22
BIO19 – Precipitation of the coldest quarter	-0.62	-0.62	0.05
Eigenvalue	7.46	4.83	2.78
% Variation	39.25	25.43	14.64

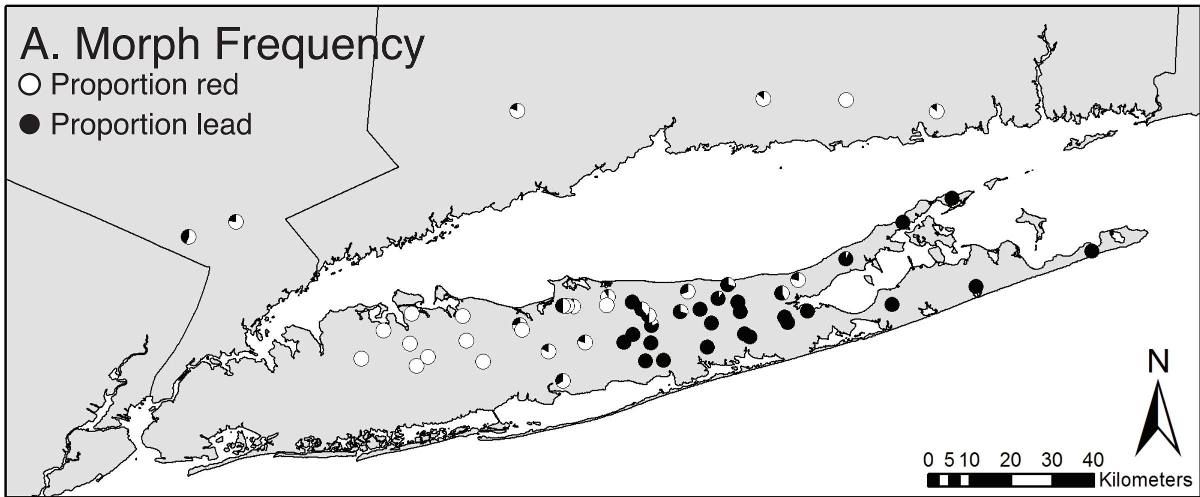
Table 3.3. Pairwise F_{ST} and geographic distances for select populations of *P. cinereus*. Below the diagonal: F_{ST}; Above the diagonal: geographic distance (in kilometers). LI = Long Island. Abbreviations for mainland localities are: CTMO = Connecticut-Monroe; CTNG = Connecticut-North Guilford; CTOL = Connecticut-Old Lyme; NYOr = New York-Orangeburg; NYSH = New York-Sleepy Hollow; NJDu = New Jersey-Dunnfield Creek.

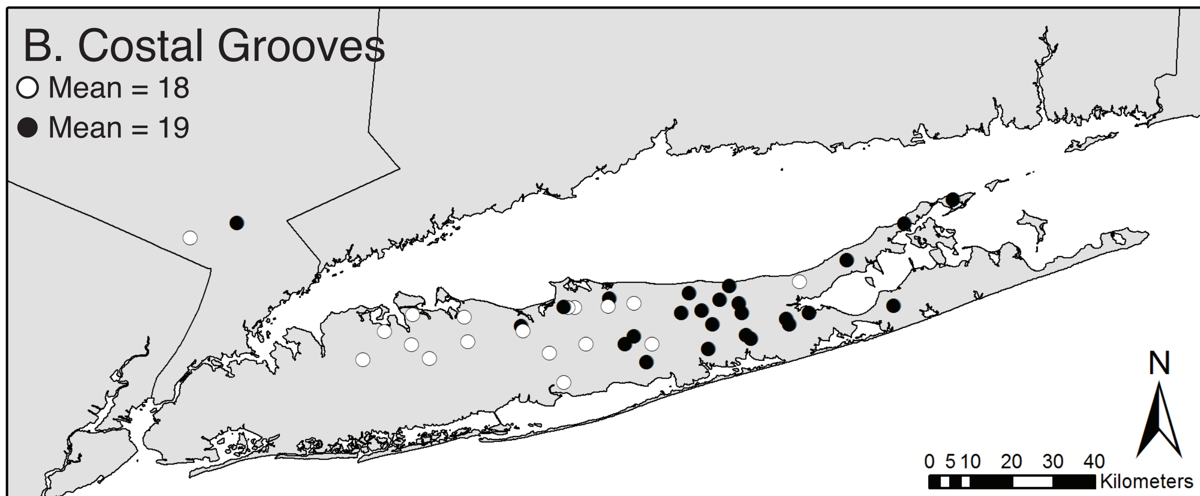
	LI pure-lead	LI mixed	LI pure-red	CTMO	CTNG	CTOL	CTTH	NYOr	NYSH	NJDu
LI pure-lead	-	31.70	78.63	65.50	16.11	40.27	22.01	143.45	132.10	275.35
LI mixed	0.107	-	46.96	35.12	31.19	70.63	49.66	111.77	100.43	243.65
LI pure-red	0.217	0.096	-	21.73	76.15	117.22	95.76	65.13	53.96	196.77
CTMo	0.191	0.172	0.131	-	59.51	101.36	79.51	79.92	68.47	211.73
CTNG	0.198	0.196	0.107	0.029	-	41.87	20.00	139.29	127.84	271.21
CTOL	0.142	0.204	0.146	0.128	0.071	-	21.87	181.03	169.59	313.01
CTTH	0.137	0.174	0.121	0.069	0.002	0.018	-	159.25	147.80	291.20
NYOr	0.125	0.147	0.194	0.097	0.136	0.176	0.097	-	11.45	132.02
NYSH	0.128	0.174	0.149	0.027	0.016	0.052	-0.034	0.055	-	143.44
NJDu	0.239	0.240	0.260	0.167	0.202	0.199	0.189	0.111	0.118	-

Table 3.4. Mantel correlation coefficients for each single and partial Mantel test of six hypotheses (see Materials and Methods). The bottom triangle (below the diagonal) reports the partial Mantel correlation between two variables while the third is held constant, while the top triangle (above the diagonal) reports the single Mantel correlation between two variables. Two-tailed significance was assessed with 10,000 permutations in the program PASSaGE. Coefficients which are significant after a Bonferroni correction (corrected α = 0.008) are indicated by bold font and an asterisk (*). The number of localities being compared for each hypothesis test is listed in the upper left corner of each hypothesis sub-table. A description of each matrix can be found in the Materials and Methods.

Relationship between F_{ST} and group membership	N = 46	F_{ST}	*group*	*geo*
	F_{ST}	-	**0.43955***	**0.31257***
	group	**0.44134***	-	0.06812
	geo	**0.31538***	-0.08118	-
Relationship between macroclimate and locality morph frequency	N = 64	*macro*	*morph*	*geo*
	macro	-	**0.15458***	**0.77996***
	morph	**0.08961***	-	**0.12687***
	geo	**0.77587***	0.01020	-
Relationship between macroclimate and genetic distance	N = 46	*macro*	*gen*	*geo*
	macro	-	**0.69092***	**0.75707***
	gen	**0.42392***	-	**0.62791***
	geo	**0.57449***	0.22195	-
Relationship between microclimate and locality morph frequency	N = 26	*micro*	*morph*	*geo*
	micro	-	0.01203	-0.0127
	morph	0.01355	-	0.11329
	geo	-0.01415	0.11346	-
Relationship between microclimate and genetic distance	N = 26	*micro*	*gen*	*geo*
	micro	-	0.05700	-0.01270
	gen	0.07996	-	**0.59085***
	geo	-0.05758	**0.59259***	-
Relationship between macroclimate and microclimate	N = 26	*micro*	*macro*	*geo*
	micro	-	-0.1099	-0.01270
	macro	-0.16693	-	**0.80193***
	geo	0.12704	**0.80548***	-

Figure 3.1. Maps of Long Island localities with (**A**) known morph frequency, (**B**) mean costal groove number, and (**C**) frequency of CTT mutation in ATPase6. For (A), white corresponds to the proportion of red morphs, and black corresponds to the proportion of lead morphs. For (B), white corresponds to a mean costal groove count of 18, and black corresponds to a mean costal groove count of 19. For (C), white corresponds to the proportion of individuals in a population that lack the CTT mutation in ATPase6, while black corresponds to the proportion of individuals who have the CTT mutation. For A and B, only localities with $N \geq 4$ are shown. For C, all localities have $N = 2$. For additional localities with morph frequency and costal groove data, see Figure 1 in Williams et al. (1968).


A. Morph Frequency
Proportion red
Proportion lead
N
0 5 10 20 30 40
Kilometers


B. Costal Grooves
Mean = 18
Mean = 19
N
0 5 10 20 30 40
Kilometers

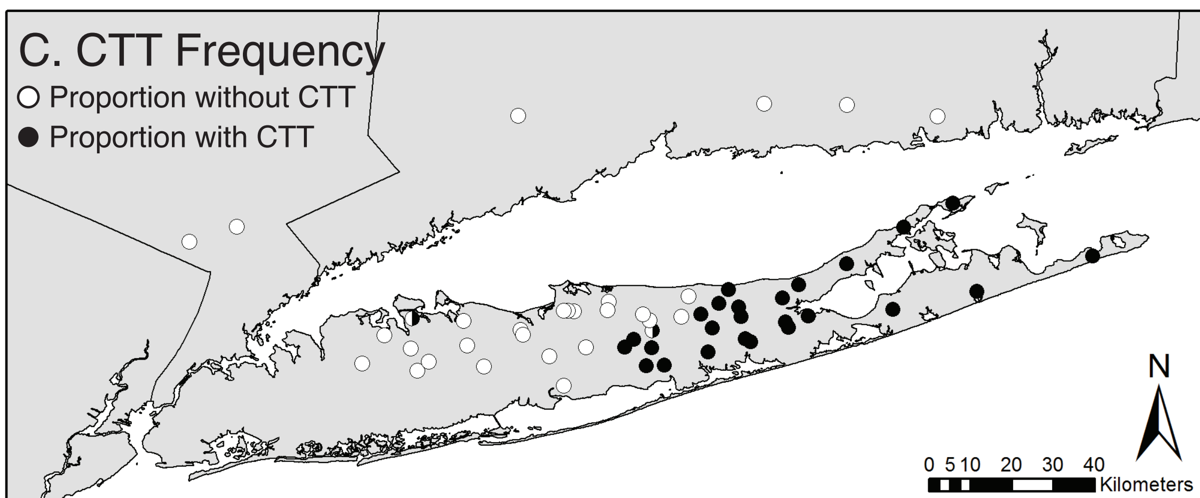
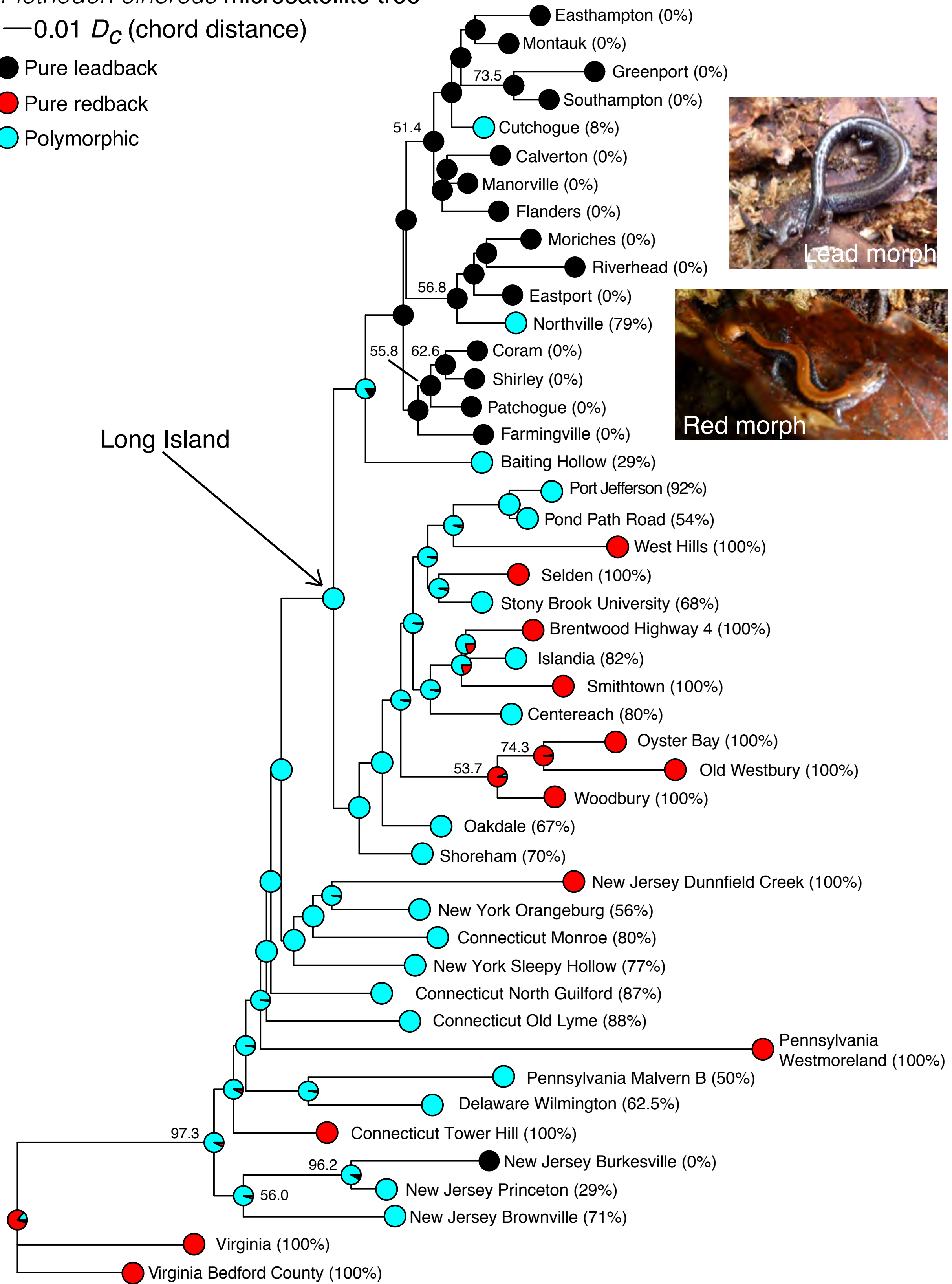
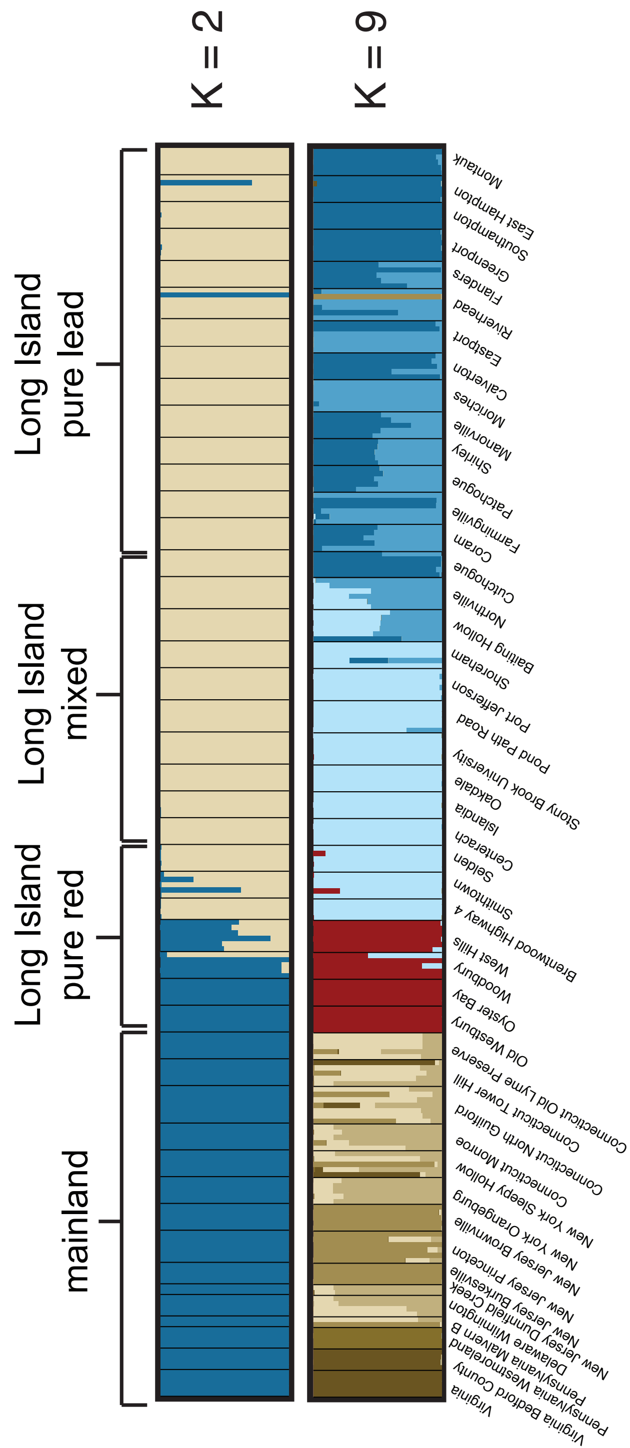

C. CTT Frequency
Proportion without CTT
Proportion with CTT
N
0 5 10 20 30 40
Kilometers

Figure 3.2. A phylogeny of *P. cinereus* populations based on microsatellite data (Fitch-Margoliash tree from chord distances). Bootstrap values < 50 are not shown. Pie charts on nodes show the results of a likelihood based ancestral reconstruction of general morph-frequency categories. The morph frequency of the locality is in parentheses following the locality name. All photos taken by MCFR.



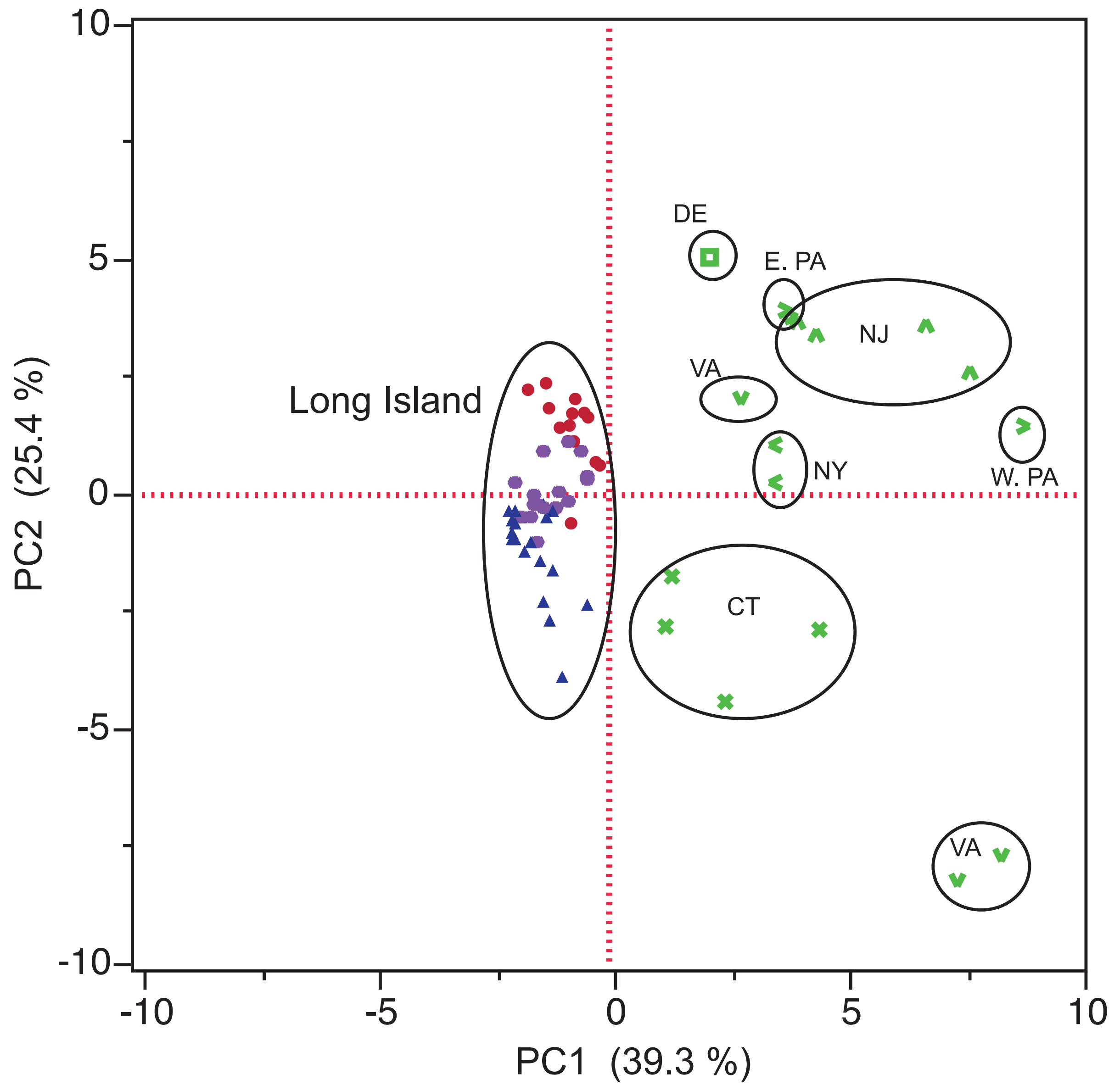
Plethodon cinereus microsatellite tree
0.01 Dc (chord distance)
Pure leadback
Pure redback
Polymorphic
Easthampton (0%)
Montauk (0%)
73.5
Greenport (0%)
Southampton (0%)
51.4
Cutchogue (8%)
Calverton (0%)
Manorville (0%)
Flanders (0%)
Moriches (0%)
Riverhead (0%)
56.8
Eastport (0%)
Northville (79%)
55.8
62.6
Coram (0%)
Shirley (0%)
Patchogue (0%)
Farmingville (0%)
Baiting Hollow (29%)
Long Island
Lead morph
Red morph
Port Jefferson (92%)
Pond Path Road (54%)
West Hills (100%)
Selden (100%)
Stony Brook University (68%)
Brentwood Highway 4 (100%)
Islandia (82%)
Smithtown (100%)
Centereach (80%)
74.3
Oyster Bay (100%)
53.7
Old Westbury (100%)
Woodbury (100%)
Oakdale (67%)
Shoreham (70%)
New Jersey Dunnfield Creek (100%)
New York Orangeburg (56%)
Connecticut Monroe (80%)
New York Sleepy Hollow (77%)
Connecticut North Guilford (87%)
Connecticut Old Lyme (88%)
Pennsylvania Westmoreland (100%)
Pennsylvania Malvern B (50%)
Delaware Wilmington (62.5%)
97.3
Connecticut Tower Hill (100%)
96.2
New Jersey Burkesville (0%)
New Jersey Princeton (29%)
56.0
New Jersey Brownville (71%)
Virginia (100%)
Virginia Bedford County (100%)

Figure 3.3. Results from Bayesian cluster analysis of the microsatellite data (using Structure), showing bar plots for the optimal K values of 2 and 9. Each single line is an individual, and each group of lines (denoted by black separator bars) is a group of individuals from the same locality (N = 46 total localities). Each genetic cluster is represented by a unique color, and the individuals are colored to represent their estimated membership fraction for each genetic cluster. For K = 2 (selected by the ΔK method; Evanno et al. 2005), there is a pure Long Island cluster of mixed and pure-lead populations, and a mainland/Long Island cluster containing mainland and pure-red populations. For K = 9 (selected by having the best log likelihood score; Pritchard et al. 2010), there are five mainland clusters and four Long Island clusters. The Long Island clusters separate largely by population morph frequency, although there is overlap between (a) pure-reds and mixed and (b) pure-leads and mixed. Note that a single Riverhead individual clusters with mainland groups in both plots. We hypothesize that this individual was transplanted to Long Island recently, via topsoil import for agriculture.



K = 2
K = 9
Long Island pure lead
Long Island mixed
Long Island pure red
mainland
Montauk
East Hampton
Southampton
Greenport
Flanders
Riverhead
Eastport
Calverton
Moriches
Manorville
Shirley
Patchogue
Farmingville
Coram
Cutchogue
Northville
Baiting Hollow
Shoreham
Port Jefferson
Pond Path Road
Stony Brook University
Oakdale
Islandia
Centerach
Selden
Smithtown
Brentwood Highway 4
West Hills
Woodbury
Oyster Bay
Old Westbury
Connecticut Old Lyme Preserve
Connecticut Tower Hill
Connecticut North Guilford
Connecticut Monroe
New York Sleepy Hollow
New York Orangeburg
New Jersey Brownville
New Jersey Princeton
New Jersey Burkesville
New Jersey Dunnfield Creek
Delaware Wilmington
Pennsylvania Malvern B
Pennsylvania Westmoreland
Virginia Bedford County
Virginia

Figure 3.4. Localities of *Plethodon cinereus* plotted in multivariate climatic niche space (PC1 vs. PC2; see Table 3.2 for loadings). Green symbols are mainland localities, with different states indicated with different shapes. Red circles are pure-red localities on Long Island. Purple circles are mixed localities on Long Island. Blue triangles are pure-lead localities on Long Island. CT = Connecticut, DE = Delaware, E. PA = Eastern Pennsylvania, W. PA = Western Pennsylvania, NJ = New Jersey, NY = New York, VA = Virginia.



PC2 (25.4 %)
PC1 (39.3 %)
Long Island
DE
E. PA
NJ
VA
NY
W. PA
CT
VA
10
5
0
-5
-10
-10
-5
0
5
10

Conculsions

Speciation, the origination of new species, is a subject at the heart of evolutionary biology (Futuyma 2009). Darwin (1859) recognized from the start that natural selection based in an organisms ecology plays a role in the splitting of lineages, and thus ecological divergence is at the heart of speciation. Together, both speciation and ecological divergence among populations and species are commonly studied under a phylogenetic framework, which permits questions about the relationships between traits and history. Thus, phylogenetic analyses have become a routine component of evolutionary and ecological studies, particularly those asking questions about speciation and the history of ecological divergence among species. In this dissertation, we closely examine the phylogenetic methods used to study speciation, as well as ask questions about the relationships between traits involved in speciation, and the processes of ecological divergence and speciation in plethodontid salamanders.

In Chapter 1, combined-data phylogenetic methods were tested for the impact of combining mitochondrial and nuclear DNA, which have very different genetic histories and patterns of inheritance. Across 14 clades of vertebrates, we found that conflict between mitochondrial and nuclear DNA data is quite common. However, these conflicts are often weakly supported statistically by one or both data sets. We find that nuclear DNA dominates the combined-data tree more often than mitochondrial DNA (even with fewer variable characters), and both data types contribute to the resolution of the combined-data tree (i.e., they resolve at different depths). These results are general across the 14 clades, excepting *Plethodon* salamanders. Despite the addition of five new nuclear loci, the *Plethodon* combined-data tree very closely matches the mitochondrial topology. *Plethodon* are known to hybridize among distantly related species (Highton 1995; Weisrock et al. 2005; Wiens et al. 2006), and so this pattern of mitochondrial dominance in the combined-data tree may be a signature of past or current hybridization. Thus, we conclude Chapter 1 by recommending that testing congruence between mitochondrial and nuclear trees be an essential precaution in combined-data studies, particularly in groups with extensive hybridization among species (like *Plethodon*).

In Chapter 2, we test for a relationship between the rate of climatic-niche evolution and climatic-niche breadth in the salamander family Plethodontidae. The climatic niche has previously been implicated in speciation (e.g., Kozak and Wiens 2006, 2010; Smith and Beaulieu

2009), and understanding how it evolves is critical for further understanding of ecological speciation, species' responses to climate change and several other topics discussed in Chapter 2. Despite previous studies implying that breadth and rate might be related (e.g., Smith and Beaulieu 2009; Kozak and Wiens 2010), we find no general relationship between the rate of multivariate climatic-niche evolution and multivariate climatic-niche breadth. However, when specific climatic variables are analyzed, a strong positive relationship does emerge for some variables (e.g., annual precipitation). This latter relationship suggests that species with broad climatic niches have climatic niches which evolve more quickly than those with narrow climatic niches, contrary to our expectations..

Finally, in Chapter 3, we analyze a potential case of parapatric divergence of sympatric morphs and incipient speciation on Long Island, in the terrestrial salamander *Plethodon cinereus*. Our results suggest that two sympatric color morphs of *P. cinereus* (redback and leadback) invaded Long Island thousands of years ago and subsequently distributed themselves parapatrically along a climatic gradient. In western Long Island, pure red populations now occur in warmer and wetter habitats in western Long Island, whereas pure lead populations occur in cooler and drier habitats in eastern Long Island. Populations in central Long Island contain both morphs. Long Island populations exhibit much stronger between-population genetic differentiation compared to mainland populations across similar geographic distances. To our knowledge, this is the first empirical case of parapatric segregation of previously sympatric morphs across a macrogeographic climatic gradient. Our data also suggest incipient speciation of the pure lead populations. These populations already experience highly restricted gene flow from other Long Island populations, and additionally show evidence suggesting local adaptation. Finally, other members of the genus *Plethodon* are polymorphic or monomorphic for the same two colors and appear to have similar ecological tolerances, suggesting that the intraspecific patterns observed here may have lead to full speciation in other species in this genus. Further study in this system will determine how important this polymorphism is to speciation within *Plethodon* and elucidate the more general process of parapatric divergence between sympatric morphs.

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