Evolutionary dynamics among salamanders in the *Plethodon glutinosus* group, with an emphasis on three species: *P. jordani*, *P. metcalfi*, and *P. teyahalee* (Caudata: Plethodontidae)

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Ecology and Evolutionary Biology)
In The University of Michigan
2009

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ACKNOWLEDGEMENTS

This dissertation would not have been possible without the help and guidance of a great many people and institutions. I would especially like to thank Priscilla Tucker for her patience and willingness to engage at every stage of this long process. Her dedication, not just to research, but education as well has made this project possible. I would also like to thank my other committee members: Ron Nussbaum, Tom Duda, and Jeff Long.

A few people have provided early insight into the study system, most notable are Richard Highton and Stephen Tilley. These acknowledgements would not be complete without mentioning Nelson Hairston, Sr. Although I only the chance to meet him once before his death last summer, his work on plethodontid biology and this study system in particular laid the groundwork for this dissertation.

The work presented here is the product of a long and expensive endeavor, and there are many institutions that have generously given their financial support. Many departments and institutions within the University of Michigan have been especially generous, including the Department of Ecology and Evolutionary Biology, the Museum of Zoology, Rackham Graduate School, and the Department of Human Genetics. Other essential granting institutions and societies include: the National Institutes of Health, the Society for the Study of Amphibians and Reptiles, and the North Carolina Herpetological Society.

Lab work was undoubtedly the largest component of this project, and the learning curve was steep. For their help, I would like to thank past and present members of the Tucker Lab, especially Les Harris for showing me the ropes of lab work. Elizabeth Jockusch gave me much needed nuclear DNA primers, and time and patience after that. David Weisrock provided help with the mtDNA primers, and his own dissertation work on the *Plethodon jordani* complex is often cited in this dissertation. I would also like to thank Ken Luzynski and Armand Conti for spending last summer genotyping samples in the lab.

Field work was not only an essential component of my dissertation work, but provided a much needed respite from urban life. For making work in the Smokies and my summers in the field an absolute pleasure, I would like to thank the crew at the Appalachian Highlands Science Learning Center at Purchase Knob, especially Paul Super, Susan Sachs, Jonathan Mays, and Gabrielle Riesner. Kevin Hamed’s help in the field is almost as appreciated as his wit and humor.

I am indebted to Ben Fitzpatrick for his help with the statistical analysis in Chapter III and Ken Kozak for creating the niche models used in Chapter III and IV. Both of these excellent researchers are rightfully co-authors. I would also like to thank Amanda Zellmer for her time and patience in helping with the niche modeling (and other projects not related to this dissertation).

For making my graduate career proceed relatively smoothly, I need to thank the staff of the Department of Ecology and Evolutionary Biology, especially LaDonna Walker, Julia Eussen, and Jane Sullivan. I also need to thank the staff at the Museum of

Zoology, especially Bev Dole, Norah Daugherty, and Robbin Murrell. Their aid in the day-to-day logistics of carrying out this project was invaluable.

I am not able to express my gratitude deeply enough to the following people: Lucía Luna for making office life not only tolerable, but fun; Matt Vance for his patience throughout; and Clay Vance for reminding me that it is the love of the organism that ultimately matters. I cannot end these acknowledgements without thanking my parents, Virginia and Robert Chatfield, for instilling the idea that one should always put education and the pursuit of one's interests above all else.

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ABSTRACT

Evolutionary dynamics among salamanders in the *Plethodon glutinosus* group, with an emphasis on three species: *P. jordani*, *P. metcalfi*, and *P. teyahalee* (Caudata: Plethodontidae)

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This dissertation includes three studies that focus on the evolution of salamanders in the *Plethodon glutinosus* group (family Plethodontidae), that are found throughout much of the eastern United States. The first study explores species delimitation in taxonomically problematic groups and then, in that context, examines the long and contentious taxonomic history of the *P. glutinosus* group. Four commonly encountered problems are reviewed: (1) the presence of cryptic species complexes, (2) incomplete lineage sorting, (3) introgressive hybridization, and (4) the application of different species concepts. The history of species description in the *P. glutinosus* group is then discussed in light of these problems. The controversial use of DNA barcoding is also discussed, as is its potential utility in the group. The second study is a spatially fine-scale analysis of a hybrid zone that occurs among three species within the *P. glutinosus* group – *P. jordani*, *P. metcalfi*, and *P. teyahalee* – in the southern Appalachian Mountains. Spatial patterns of variation along four transects are examined at four markers: single nucleotide polymorphisms located in the mtDNA ND2 gene and the nuclear DNA ILF3 gene and the morphological markers of red cheek pigmentation and white flecks. Concordance among nuclear DNA and both morphological markers across the four transects is observed. In three of the four transects, however, the pattern of mtDNA is discordant from all other markers. This finding, in addition to previous studies demonstrating mating asymmetry and differing ecological niches, suggests the hybrid zone may be moving. The third study uses ecological niche modeling to explore range dynamics of *P. jordani* and *P. metcalfi* at three time periods: (1) present-day, (2) during the last glacial maximum 21,000 years ago, and (3) in the year 2050 under a climate warming scenario. Generated models suggest the distributions of these species are influenced by precipitation and are dynamic across the time periods studied. The conservation status of *P. jordani* and *P. metcalfi* are discussed in light of anthropogenic climate change.

CHAPTER I

Introduction

Arnold et al. (1993) expressed a growing sentiment when they stated that the family Plethodontidae is an ideal system in which to study the evolutionary process of speciation. For no group within Plethodontidae is this more true than for the *Plethodon glutinosus* group. The range of this monophyletic group extends throughout eastern North America, from the lowest to the highest elevations (Petranka 1998). Ecological studies within the group have a long history, making it relatively well known and amenable as a model system (Arnold et al. 1993). The taxonomic history of the group has a long history as well, extending back to the early 1800s (Gilliam 1818, Green 1818), but it is only in recent decades, with the advent of molecular tools, that species boundaries and relationships have been refined (Highton 2000), and ecological and evolutionary processes understood.

Research on the evolution of the group continues to document extensive hybridization. Based on morphology and allozyme data, 28 lineages are currently recognized as species (Highton 1989, Highton and Peabody 2000). Among these, 17 pairs are sympatric and five are known to hybridize; and among 16 parapatric pairs, 11 are known to form hybrid zones (Highton and Peabody 2000). Detailed studies of hybridizing taxa, especially using genetic markers, have revealed a complex biogeographic history and numerous instances of introgressive hybridization (Highton

1970, 1972, 1989, Hairston et al. 1992, Weisrock et al. 2005, Weisrock and Larson 2006). This is most readily seen in studies in the southern Appalachians, where mountainous terrain, climate change, and niche conservatism (Kozak and Wiens 2006) have led to numerous mountain-top isolates.

Three species within the *P. glutinosus* group – *P. jordani*, *P. metcalfi*, and *P. teyahalee* – are especially well studied. These species were the focus of much of Hairston's (Hairston 1980a, 1980b, 1983, Hairston et al. 1987) ecological work on interspecific competition. His findings suggest that competition likely plays a role in species distributions. Hybridization among the species was known as far back as the middle of the last century when morphological intermediates between *P. jordani* and *P. metcalfi* (Hairston 1950) and *P. jordani-P. metcalfi* hybrids and *P. teyahalee* (Highton 1970) were first reported. Numerous studies since then have provided information on the geographic extent and relative abundance of hybrids. For example, using allozymes, Peabody (1978) examined differences among the species. His cursory analysis of hybridization between *P. jordani* and *P. teyahalee* documented, for the first time, the presence of hybrids using genetic markers. Manzo (1988) conducted a morphometric analysis of *P. jordani* and *P. teyahalee* and concluded that the parental species were different from one another and that hybrids exhibited intermediacy. Lastly, Reagan (1992) studied sexual isolation between populations of *P. jordani*, *P. metcalfi*, and *P. teyahalee*. Her study documented asymmetrical mating between many different populations, including *P. jordani* and *P. metcalfi*. Furthermore, the study revealed variation in the extent of reproductive isolation between *P. jordani* and *P. teyahalee*, with some populations exhibiting greater isolation than others. These findings are interesting in that they suggest different regions of hybridization may have different evolutionary dynamics.

Species delimitation is at the heart of the fundamentally important question, “What is a species?” The answer to this question has important implications for many fields within biology, including systematics, ecology, behavior, and evolution (Mayr 1963, Highton 1995, Cracraft 2002, King et al. 2008). Nearly all these studies use species as the fundamental unit of classification (Harrison 1998), making accurate species boundaries essential to uncovering biological patterns. Lastly, the urgent need to catalogue biodiversity in the face of impending extinctions (Balakrishnan 2005), as well as identifying distinct evolutionary lineages for conservation purposes (Rojas 1992, Cracraft 2002, Hey et al. 2003, Agapow et al. 2004, Mace 2004) makes species delimitation even more important.

Chapter II of this dissertation begins with a review of many of the problems encountered when delimiting species boundaries. Four of these are discussed at length: (1) the use of different species concepts, (2) the presence of cryptic species complexes, (3) incomplete lineage sorting, and (4) introgressive hybridization. Numerous examples are presented of studies that have encountered these issues, and a brief summary of how each problem is combated is also given. Included in this chapter is a discussion on the utility of DNA barcoding. This is a controversial topic that has received considerable attention in recent years (Rubinoff et al. 2006, Hajibabaei et al. 2007).

The second part of Chapter II discusses the role that each of the four species delimitation problems have played in the *P. glutinosus* group. Using a timeline of taxonomic history, methodological patterns of species delimitations are described throughout the long taxonomic history of the group. The chapter concludes by highlighting the following: (1) homoplasy and morphological stasis have resulted in cryptic species complexes, (2) a rapid and recent radiation has resulted in incomplete lineage sorting, and (3) introgressive hybridization resulting from incomplete reproductive isolation is common. Furthermore, the application of many different species concepts has caused considerable confusion in defining species boundaries as well. Lastly, it is unlikely that DNA barcoding will prove to be a useful tool in identifying species in this group.

Despite the fact that hybrid zones cause practical and philosophical complications in traditional taxonomic classificaton (Harrison 1990), many researchers believe we are in the midst of a paradigm shift as zoologists increasingly recognize the constructive role of hybridization in evolution (Arnold 2006, Arnold and Meyer 2006, but see Coyne and Orr 2004). There are two reasons for this: First, there is widespread recognition that hybridization among animals is more common than once thought (10% of animal species by one estimate, Mallet 2005), and second, evolutionary studies of hybridization are documenting substantial introgression (e.g., Bull et al. 2006, Minder and Widmer 2008). Introgression of adaptive alleles across a porous species boundary (Kim and Rieseberg 1999, Martinsen et al. 2001, Martin et al. 2006) represents a contrasting view to traditional models of evolution where de novo mutations are the sole source of novel adaptations in naïve populations.

As recognition of the importance of hybridization increases, so too do the ramifications of hybridization research. The clearest example of this is work being done on the evolutionary processes underlying speciation. In contrast to early proponents of the biological species concept, which originally precluded the existence of any gene flow among species (Mayr 1963), the possibility of gene flow between diverging and even full-fledged species is now commonly accepted (e.g., Kronforst 2008). Theoretical models of sympatric and parapatric speciation abound in the literature (e.g., Takimoto et al. 2000, Gavrilets and Waxman 2002, Almeida and de Abreu 2003, Kawata et al. 2007) and the number of plausible empirical examples is growing as well (e.g., Friesen et al. 2007, Herder et al. 2008). Besides evolutionary biology, hybridization research is important in the field of systematics. Increasingly sophisticated methods of species delimitation no longer require complete reproductive isolation, but rather strive to detect signals of speciation despite incomplete lineage sorting and gene flow (e.g., Knowles and Carstens 2007). Another field which is impacted by hybridization studies is conservation biology. As currently described in the Endangered Species Act, determination of protection status and the setting of conservation guidelines are dependent upon the "purity" of the taxon in question (O'Brien and Mayr 2001). The identification of natural and anthropogenically-mediated hybridization necessarily causes confusion (Allendorf et al. 2001).

Chapter III is an analysis of a hybrid zone involving *P. jordani*, *P. metcalfi*, and *P. teyahalee*. This study builds on earlier studies that have examined hybridization between *P. jordani* and *P. metcalfi* (Peabody 1978, Hairston et al. 1992) and between *P. jordani* and *P. teyahalee* (Manzo 1988, Reagan 1992). The analyses presented here expand on earlier studies in three ways: (1) patterns of spatial variation are examined for mtDNA, nuclear DNA, and morphological markers; (2) hybrid populations involving *P. jordani*, *P. metcalfi*, and *P. teyahalee* are analyzed across four transects, two along high elevation ridgelines predominately connecting the ranges of *P. jordani* and *P. metcalfi*, and two elevational transects connecting hybrid populations of the former species with that of *P. teyahalee*; and (3) sampling in this study was performed at a spatially fine scale, allowing for increased resolution in the detection of introgression.

In the discussion, the hypothesis of hybrid zone movement is put forward as a likely explanation for the pattern of differential introgression observed in this study. Support for the hypothesis of hybrid zone movement is drawn from Reagan's (1992) work on mating asymmetry. Support also comes from the ecological niche modeling presented in Chapters III and IV. Although speculative, the possibility is presented that the unique morphological character of red cheek pigmentation of *P. jordani* may play a role in the movement of the hybrid zone as well. This trait is putatively under positive selection (Huheey 1960, Brodie and Howard 1973, Hensel and Brodie 1976), and hybrid zone movement appears to be occurring from *P. jordani* into the ranges of *P. metcalfi* and *P. teyahalee*.

In Chapter IV, ecological niche modeling is used to address range dynamics in *P. jordani* and *P. metcalfi*. Models for three time periods are presented: (1) the last glacial maximum, which occurred 21,000 years ago; (2) present-day; and (3) the year 2050, using a standard scenario of climate change based on anthropogenic carbon emissions. When considered together, these models reflect the extremes of range shifts and put the hybrid zone analysis of Chapter III into a spatial and temporal context. Models generated using present-day bioclimatic variables indicate that *P. jordani* and *P. metcalfi* currently occupy nearly all of the regions that are predicted to contain suitable habitat. Models that reconstruct distributions during the last glacial maximum show little change from the present-day, although the resolution at which these models were created may be an issue. Lastly, future projections indicate that the ranges of *P. jordani* and *P. metcalfi* will be substantially smaller than present-day as the species migrate upslope to track shifts in suitable habitat.

Chapter V consists of concluding remarks on the dissertation and suggests future directions of research into the evolutionary dynamics of the *P. glutinosus* group. Avenues of research that would be especially interesting are range shifts and the changing dynamics of hybridization among *P. jordani*, *P. metcalfi*, and *P. teyahalee* in the face of anthropogenic climate change. Recent work by Highton (2005) suggests that *Plethodon* across the eastern U. S. may be experiencing severe declines. While the root cause of these declines remains unknown, numerous factors have been implicated and likely act in concert (Blaustein and Kiesecker 2002, Stuart et al. 2004, Halliday 2008, Pounds and Coloma 2008). These include deforestation (Petranka et al. 1993, Ash 1997, Harpole and Haas 1999, Duguay and Wood 2002), road construction (Semlitsch et al. 2007), and infection by the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (Vazquez et al. 2009). Addressing the role of anthropogenic climate change in the fate of these three species will require expanding on both the hybrid zone analysis in Chapter III and the niche modeling presented in Chapter IV to examine habitat preferences, changing habitats, and range dynamics at a fine scale. The work presented in this dissertation is a first step in the right direction.

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CHAPTER II

Difficulties with species delimitation and DNA barcoding in the *Plethodon glutinosus* group (Caudata: Plethodontidae)

Abstract

Species delimitation is a fundamental component of systematics, the accuracy of which is essential to many fields including behavior, ecology, evolutionary biology and conservation. Given this importance, numerous studies have elucidated the difficulties in accurate species delimitation. This review is a synthesis of four commonly encountered problems: (1) the presence of cryptic species complexes, (2) incomplete lineage sorting, (3) introgressive hybridization, and (4) the application of different species concepts. Some, often all, of these problems are encountered through the use of DNA barcoding. Barcoding relies on a short segment of mtDNA to describe or identify species, often (but not always) in conjunction with other data (e.g., additional mtDNA, nuclear DNA, allozymes, morphology, geography). Criticism of barcoding is pervasive in the literature; however, proponents point to the utility of a single, universal, easily-applied molecular tool for species description and identification. Arguably, few species groups have a longer and more contentious taxonomic history than the *Plethodon glutinosus* group. Beginning with the initial description of *P. glutinosus* sensu stricto in 1818, 35 lineages (species or subspecies) have been recognized, all occurring in the eastern United States. Currently, 28 species are commonly accepted. The history of species description

in the *P. glutinosus* group has been contentious, involving numerous name changes and disagreements. Much of this controversy results from: (1) homoplasy and morphological stasis, resulting in cryptic species complexes; (2) rapid, recent radiation resulting in incomplete lineage sorting; and (3) introgressive hybridization from a lack of complete reproductive isolation. Furthermore, the application of many different species concepts has caused considerable confusion in defining species boundaries as well. Recently, great strides have been made to combat these problems, but it is unlikely that DNA barcoding will prove to be a useful tool in identifying species in this group.

Introduction

Species delimitation, how species boundaries are described and new species identified, is a major topic within the field of systematics (Cracraft 2002, Wiens 2007). This is partly due to the nature of the field of systematics, which is charged with addressing the question "What is a species?" (Cracraft 2002). Additionally, accurately delimiting species into real and natural groups that are biologically meaningful is essential for basic ethological, ecological, and evolutionary studies (Mayr 1963, Highton 1995, Cracraft 2002, King et al. 2008). Nearly all these studies use species as the fundamental unit of classification (Harrison 1998), making accurate species boundaries essential to uncovering biological patterns. Lastly, the urgent need to catalogue biodiversity in the face of the extinction crisis (Balakrishnan 2005), as well as identifying distinct evolutionary lineages for conservation purposes (Rojas 1992, Cracraft 2002, Hey et al. 2003, Agapow et al. 2004, Mace 2004) makes species delimitation even more important.

Despite its importance, accurate species delimitation is fraught with difficulties. Methodologies that (1) yield consistent results, (2) are universally applicable (i.e., may be applied irrespective of the taxon under consideration), and (3) are easily applied across different types of data sets (e.g., single nucleotide polymorphisms, DNA sequences, allozymes, morphology) remain elusive and have not been thoroughly tested (Balakrishnan 2005). Other difficulties hindering accurate species delimitation deal not with the development of appropriate methods, but rather with our understanding of lineage independence or the populations themselves. These include the presence of cryptic species complexes, incomplete lineage sorting, and introgressive hybridization. These difficulties are in addition to the basic supposition that the lineages under consideration are well sampled (Funk and Omland 2003). Lastly, there is the theoretical issue of what constitutes a species.

One controversial topic that has received considerable attention in recent years is DNA barcoding. Using this technique, researchers attempt to streamline species identification and description through the sequencing of standard mtDNA sequences, often in conjunction with more traditional (i.e., integrative) systematic approaches (Rubinoff et al. 2006, Hajibabaei et al. 2007). Proponents stress the easy application of barcoding by non-molecular biologists, the speed at which species can be identified, and the standardized results making cross-species comparisons relatively easy (Hebert et al. 2004). Criticism of barcoding has been extensive and ranges from claims of insurmountable technical difficulties to oversimplification of systematic inquiry that may ultimately undermine the integrity of systematic research (Rubinoff et al. 2006). Both sides of the argument seem to agree, however, that some degree of error in species classification is inevitable (<5%, see Hajibabaei et al. 2007; ~20%, Meyer and Paulay 2005). Taxa that are most prone to misclassification are likely to be the same as those for which traditional systematics have been difficult, i.e., in the presence of incomplete lineage sorting and introgressive hybridization.

Certain taxa have proved especially contentious in attempting to delimit species boundaries. The *Plethodon glutinosus* group (family Plethodontidae) in the southeastern United States is one such example. Species description in this group began in the early 1800s and extends to the 21st century. Current recognition of 28 species in the group (Highton and MacGregor 1983, Highton 1983, 1989, Highton and Peabody 2000) is gaining acceptance, although the methods by which much of this diversity was described has been debated (see Frost and Hillis 1990 and Highton 1990). The long history of species delimitation in this group highlights many difficulties in species delimitation in other groups of organisms, and understanding the root of these difficulties will help us understand the nature of species boundaries.

Methods for delimiting species have recently been reviewed (Sites and Marshall 2003, 2004) and will not be discussed here. Rather, the objective of this paper is to review some of the problems associated with delimiting species among lineages that have recently speciated or are in the process of speciating. These problems are clearly and sometimes integrally related to one another. As a result, many researchers will find themselves facing many or even all of these difficulties. The *P. glutinosus* group is an especially good example of the difficulties that can arise in species delimitation. Systematists delimiting species in this group have had to contend with the presence of cryptic species, incomplete lineage sorting, introgressive hybridization, and the application of different species concepts. Also presented is a comprehensive taxonomic review, which further highlights the ambiguity of species boundaries and relationships in this group. Lastly, the deficiencies of DNA barcoding as a method of species delimitation for this group are apparent and will be discussed.

Difficulties with species delimitation

Different species concepts

There are over 20 different species concepts discussed in the literature (see Harrison 1998, de Queiroz 1998, and Coyne and Orr 2004 for partial reviews). These conceptual groups fall into two categories, those that are based on reproductive isolation and those based on phylogenetic clustering. Within these two broad conceptual categories, the criteria used to delimit species boundaries may vary considerably. Different conclusions on species delimitation may be drawn depending on which species concept a researcher chooses (e.g., Young 1998, Agapow et al. 2004, Sanders et al. 2006, Alstrom et al. 2008). For example, when comparing two of the most common concepts, the Biological Species Concept (BSC; sensu Mayr 1963) and the Phylogenetic Species Concept (PSC; sensu Cracraft 1989), Dettman et al. (2003) found that when using the PSC they recognized five independent lineages in a group of 73 samples from the fungus *Neurospora*, while using the BSC they recognized only four lineages. The discrepancy suggests two possible causes: (1) phylogenetic divergence precedes reproductive isolation (Dettman et al. 2003) or (2) the PSC offers greater resolution and increased ability to distinguish independent lineages (Dettman et al. 2003, Agapow et al. 2004). Similarly, Gleason et al. (1998) obtained conflicting phylogenies when comparing the

BSC to the PSC in a study of the *Drosophila willistoni* group. In contrast to Dettman et al. (2003), however, the BSC recognized more lineages than the PSC (Gleason et al. 1998). Studies in which more species are diagnosed using reproductive isolation criteria (such as the BSC) than phylogenetic criteria (such as the PSC) are uncommon. In a review of 83 published studies (incorporating plants, fungi, invertebrates, vertebrates, and some microorganisms), Agapow et al. (2004) found that variants on the PSC recognized 48.7% more species than the BSC and other non-phylogenetic methods.

Despite the studies comparing phylogenetic and reproductive isolation criteria, which are relatively few in number, researchers rarely state explicitly the species concept to which they adhere. This oversight makes it difficult to determine the full extent of the disagreements among species concepts. Furthermore, studies comparing different methods have done so with two, or occasionally three, different species concepts, a small number relative to the number of concepts in existence.

Although no species concept excels under all circumstances, the BSC and the PSC have both been severely criticized. From a philosophical perspective, the BSC is unsatisfactory to many because, by relying on reproductive isolation, it does not always yield monophyletic groups, and therefore does not reflect evolutionary history (Harrison 1998). From a practical standpoint, determining whether or not two populations can and do interbreed in the wild is just not feasible. This is especially problematic when the populations under consideration are allopatric. Furthermore, it is increasingly apparent that hybridization in nature is more common than once thought (25% of plant species and 10% of animal species, Mallet 2005). The reproductive barrier between hybridizing populations can range from weak to nearly complete, making species delimitation among hybridizing taxa seem arbitrary. Lastly, the BSC is inoperable for extinct and asexual taxa (see Agapow et al. 2004). The PSC, likewise, has been criticized. Coyne (1994) offers a philosophical drawback of the PSC because it neither yields insight nor promotes research into speciation and the maintenance of species boundaries. There are also notable operational problems with the PSC. For instance, because of incomplete lineage sorting and introgressive hybridization (see below), gene trees do not always equate to species trees, making species diagnosis imperfect (see Coyne and Orr 2004).

Attempts have been made to reconcile the differences among competing species concepts and the problems that result when researchers apply different criteria to species delimitation. de Quieroz (1998) proposed the General Lineage Concept (GLC), which states that “all contemporary species definitions describe variations of the general concept of species as evolutionary lineages”. This concept, therefore, seeks to define the underlying fundamental similarity among all other species concepts, namely, the identification of independent population level evolutionary lineages. de Quieroz (1998) emphasizes that speciation is a process and, as such, contains a “gray zone” of multiple “events” that occur throughout this process. All other species concepts, the author argues, simply place a species boundary at one of these events, creating a threshold. From the conceptual point of view of the GLC, competing species concepts are, in fact, complementary.

Cryptic species

Recognition of species boundaries may be difficult in taxa where morphological stasis or homoplasy (Wake 1991, Mueller et al. 2004) result in an initial underrepresentation of the true extent of species diversity. There is an increasing number of studies where the full extent of diversity is revealed only after the application of molecular methods. For example, using mtDNA and AFLP data, King et al. (2008) discovered abundant cryptic diversity in British lumbricid earthworms. In one purported species, *Allolobophora chlorotica*, the authors identified 55 mtDNA haplotypes representing five highly divergent lineages that likely represented multiple cryptic species. In another example, the plethodontid salamander *Desmognathus ochrophaeus* was thought to be monotypic until allozyme studies revealed substantial cryptic diversity. Tilley et al. (1978), in a preliminary analysis using 11 allozyme loci, found significant genetic differentiation among para- and allopatric populations of what was then considered to be a single species, *D. ochrophaeus*. Using an expanded dataset of 22 allozyme loci and additional collecting localities, Tilley and Mahoney (1996) and Anderson and Tilley (2003) identified five lineages. Applying the Evolutionary Species Concept (a variant of the PSC that aims to identify independent evolutionary lineages), the authors elevated all five lineages to species status. Kozak et al. (2005), using mtDNA, included multiple species from the *D. ochrophaeus* complex in a genus-wide phylogenetic analysis. Results from this study suggest that, not only is the *D. ochrophaeus* complex non-monophyletic, but some species within the complex are also non-monophyletic.

Problems associated with delimiting species in cryptic species complexes are already being tackled. This is done principally through the increased use of molecular phylogenetics (see Wake 2006), and is one area where DNA barcoding has proved especially useful (e.g., Hebert et al. 2004). As originally proposed, and often emphasized, barcoding performs best when used in addition to other systematic approaches, such as the use of multiple markers, nuclear markers, morphology, etc. (Hebert et al. 2004, Rubinoff et al. 2006). Barcoding in this broader integrative approach becomes just one tool of many, and is often not the primary mode of species delimitation. One proposed use of barcoding is to perform preliminary scans of known cryptic species complexes in order to best allocate research effort (Rubinoff et al. 2006).

The use of a genetic cutoff is an inherent component of DNA barcoding (Rubinoff et al. 2006); however, the use of a genetic threshold to delimit species boundaries has been around much longer. Using allozymes, Baverstock et al. (1977) found that rodent populations that were fixed at 15% of their loci (Nei's genetic distance, *D*, of 0.15) were likely to have achieved species status. Similarly, Thorpe (1982) estimated that the threshold necessary for allopatric populations to be considered separate species is a genetic identity of 0.85, which equates to a Nei's *D* of 0.16. Thorpe (1982) continues by stating that using genetic distance is warranted when the populations in question are allopatric. The controversy surrounding the use of genetic cutoffs reached a pinnacle when Highton (1989) used a cutoff of a Nei's *D* of 0.15 to delimit species in the *P. glutinosus* group (see below, as well as Frost and Hillis 1990 and Highton 1990).

Incomplete lineage sorting

Lineage sorting is the gradual loss of ancestral polymorphisms, which results in monophyletic groups (Funk and Omland 2003). Difficulties in species delimitation arise when researchers attempt to infer a species tree based on one or more gene trees that were constructed using incompletely sorted genes. Such situations are likely to occur when lineages have only recently diverged (Shaffer and Thomson 2007). Differentiating incomplete lineage sorting from introgressive hybridization, which may create a similar pattern of shared polymorphisms, can be difficult. Oftentimes, information from many independent markers (Weisrock et al. 2006), the geographic extent of shared polymorphisms (Moran and Kornfield 1993), the frequency of hybridization (Moran and Kornfield 1993, Pinho et al. 2008), and the age of shared alleles (Pinho et al. 2008) is required to distinguish between these two phenomena.

One of the most well known examples of incompletely sorted lineages comes from cichlid fishes of Lake Malawi. Based on known dates of lake level fluctuations, many of the 300 species of Lake Malawi cichlids are only about 25,000 years old, yet many species have diverged substantially both ecologically and behaviorally (Albertson 2008). A mtDNA phylogeny constructed for this group, however, fails to reflect what is believed to be the true species relationships, with many species being polymorphic for divergent haplotypes (Moran and Kornfield 1993). Incomplete lineage sorting, as opposed to introgressive hybridization, is the most likely explanation for the observed pattern because: (1) hybrids have never been recorded for this well studied group, (2) interspecific courtship has never been observed, and (3) shared polymorphisms are widespread across species ranges and not restricted to areas of overlap or parapatry (Moran and Kornfield 1993).

As another example, *Podarcis* lizards of the Iberian Peninsula and North Africa constitute a cryptic species complex, with mtDNA sequence divergences (8-12%) greater than those used to define other squamate species (2-5.4%) (Pinho et al. 2007). Although there are currently four described species, Pinho et al. (2007) found evidence for 11 diagnosable lineages. In a recent study of *Podarcis,* Pinho et al. (2008) compared two nuclear gene phylogenies to previously published mtDNA and allozyme phylogenies. Despite general agreement between the mtDNA and allozyme phylogenies, the nuclear DNA phylogenies are incongruent with mtDNA and allozymes. Again, explaining this incongruence requires distinguishing between the competing hypotheses of incomplete lineage sorting and hybridization. The authors give three lines of evidence in favor of incomplete lineage sorting. First, we would not expect the allozyme data to show differentiation among lineages if gene flow were causing incongruent nuclear DNA phylogenies. This was not the case, as mtDNA and allozymes did differentiate (and were in agreement) among lineages. Second, if hybridization was responsible for the observed incongruence, then we would expect both ancestral and derived alleles to be about equally widespread across species. This is not what is found, however, as more ancestral than derived alleles are shared among lineages, suggesting incomplete lineage sorting is responsible for the observed polyphyly. Third, results from coalescent modeling suggest historical gene flow is nearly absent, making hybridization unlikely.

Great strides have recently been made in resolving the problems of incomplete lineage sorting when determining species boundaries. For example, Knowles and Carstens (2007) presented a method for delimiting species that have not yet achieved reciprocal monophyly. Most species delimitation criteria use gene trees as a proxy for species trees and utilize a somewhat arbitrary threshold to determine species boundaries. Knowles and Carstens (2007) used a model-based approach to detect signals of species divergence irrespective of exclusivity criteria, which may arrive at incongruent species trees. Such an approach is important in taxa that are in the early stages of speciation as the time it takes to achieve reciprocal monophyly can be long (9-12 N generation for 95% of sampled nuclear loci to reach reciprocal monophyly, where N is equal to the historical effective population size; Hudson and Coyne 2002).

Hybridization

The role of hybridization in adaptive evolution has received considerable attention in recent years. Rates of naturally occurring hybridization are estimated to be at least 25% for plants and about 10% for animals (Mallet 2005). With such prevalence in nature, the effects of hybridization on evolutionary processes, such as speciation, are potentially great. While hybridization was traditionally thought to result in unfit hybrids that are evolutionary dead ends, researchers are increasingly viewing hybridization as a positive force, introducing new adaptations to naïve populations (see Mallet 2005 for a review).

Like incomplete lineage sorting, incomplete reproductive isolation leading to introgressive hybridization may also result in ambiguous species boundaries. There is mounting evidence that the process of speciation is usually not instantaneous, and may involve prolonged periods of hybridization. For example, Osada and Wu (2005) compared 345 coding and 143 intergenic DNA sequences between humans and chimpanzees, and concluded that divergence times among coding sequences are longer than those for intergenic sequences. This result is expected if coding sequences are more likely to contain genes that contribute to reproductive incompatibility. No such inhibitions are predicted for intergenic (i.e., neutral or nearly neutral) sequences. In a review of bird species, Price and Bouvier (2002) found that after diverging for 7-17 million years many interspecific crosses are still able to produce fertile offspring, and after 11-55 million years some viable hybrids are still produced.

In a study of Mexican salamander species in the genus *Ambystoma*, Weisrock et al. (2006) found discordance between nuclear and mtDNA gene trees. While incomplete lineage sorting may account for some of the observed non-monophyly, the authors indicate that hybridization is likely important in one group of *A. ordinarium*. The authors' thorough examination of multiple nuclear and mtDNA genes makes possible the conclusion that hybridization is causing the pattern of discordance between nuclear and mtDNA.

The *Plethodon glutinosus* group

The *Plethodon glutinosus* group belongs to the family Plethodontidae, the largest, most widespread and ecologically diverse of all salamander families. The *P. glutinosus* group, one of four groups in the genus, is monophyletic (Wiens et al. 2006). Membership in the group has ranged from one (Gilliam 1818, Green 1818) to 35 species or subspecies (Adler and Dennis 1962, Highton 1995, Highton and Peabody 2000, Wiens et al. 2006), with increases through time corresponding to increased sampling and advances in marker development and technology. Current (and widely accepted) estimates place the number of species in the group at 28 (see Highton 1989, 1995, Highton and Peabody 2000 and Wiens et al. 2006), with subdivisions consisting of the *P. glutinosus* complex (16 species), the *P. jordani* complex (seven species), the *P. ouachitae* complex (three species), and three other species (*P. kentucki*, *P. petraeus*, and *P. yonahlossee*) (Table

2.1). Subspecies are not currently recognized by most researchers (Highton and Peabody 2000).

Highton (1995), using a molecular clock applied to allozyme data, estimated that the *P. glutinosus* group underwent a rapid radiation about 5 million years ago (mya) in eastern North America. Using two nuclear and two mtDNA markers, Wiens et al. (2006) estimated the age of the radiation to be 8-14 my, and Kozak et al. (2006), using only mtDNA, estimated the radiation to have occurred 8-11 mya. Despite the age of the radiation, however, the *P. glutinosus* group exhibits the same extensive morphological stasis seen in many other groups of plethodontids (e.g., *Batrachoceps* and *Ensatina*; Wake et al. 1983, Wake 2006; see also Mueller et al. 2004).

Although the group is widely distributed across the eastern United States (Petranka 1998), the southern Appalachian Mountains are especially species rich and show greater levels of genetic diversity than surrounding areas (Marshall and Camp 2006). This is one line of evidence that has led to the suggestion that the family Plethodontidae originated in the southern Appalachians (Wilder and Dunn 1920, Beachy and Bruce 1992, but see Reuben and Boucot 1989). Southern Appalachian ecosystems, while not encompassed within the range of Pleistocene glaciers, were nonetheless directly impacted by glacial and interglacial events (see Watts 1980, Delcourt and Delcourt 1998, Crespi et al. 2003).

Most of the species' ranges in the *P. glutinosus* group are para- or allopatric, often with species boundaries coinciding with geographic boundaries such as rivers, or replacing one another elevationally. Of 17 sympatrically distributed pairs of species in the group, five are known to hybridize; of 16 parapatrically distributed pairs, 11 are known to hybridize and another four pairs are data deficient (Highton and Peabody 2000). This rampant hybridization may be viewed as multiple (mostly) independent snapshots into the speciation process and, as a result, has been called an ideal system in which to study the process of speciation (Arnold et al.1993).

Taxonomic history of the *Plethodon glutinosus* group

The taxonomic histories of many groups, especially those containing cryptic species complexes, can be partitioned into three phases. Before the widespread application of molecular methods, species were generally delimited using morphological characters. In the 1970s, it was discovered that lineages could be differentiated using allozymes (protein variants). Although still in use today, allozyme studies have been all but supplanted through the use of DNA sequence data. A detailed taxonomic history of the *Plethodon glutinosus* group is presented in Table 2.2. This history parallels the general pattern of species delimitation seen in other groups, and can likewise be partitioned into three discrete phases (Table 2.3). Phase 1, which began with the description of *P. glutinosus* in 1818, lasted until 1962. This phase is characterized by the use of morphology in the description of new species. During Phase 2, which lasted from 1978 to 2000, species were described using allozymes. The majority of new species descriptions, as well as the placement of species into monophyletic groups, took place during this phase. The contributions by one researcher, Richard Highton, dominated the allozyme phase, with nearly all contributions having been made by him and his collaborators (Highton 1962, 1989, 1995, 2000, Duncan and Highton1979, Highton and MacGregor 1983). Phase 3 began in 2005 with a publication by Weisrock et al. (2005)

utilizing mtDNA sequences to address evolutionary questions on gene flow and introgression among previously described species in the *P. glutinosus* group. Phase 3 continues to the present day.

The *P. glutinosus* group, like other plethodontid salamander groups, is known to contain cryptic species and is characterized by morphological stasis (Highton and Peabody 2000). As a result, taxonomy before the molecular age was unstable. During the morphological phase (1818-1962), 29 studies which dealt with the subject of taxonomy and/or systematics in the group were published. In the studies, 20 lineages were described (or placed under a different name, for example when a subspecies was later elevated to species status) that, at least by their authors, warranted specific or subspecific status. Fully 15 of these 20 described lineages were departures from a previous author's assigned name or systematic status (Table 2.3). In the allozyme phase (1978-2000), which lasted 22 years (versus the 144 years of the morphological phase), nine studies were published examining taxonomy and/or systematics in the group. In these studies, 20 lineages were described, all of which resulted from splitting apart cryptic species complexes into multiple species. Interestingly, there were only two disagreements in the literature amidst this taxonomic reorganization: the recognition that *P. longicrus* is not distinct from *P. yonahlossee* and the resurrection of *P. kentucki* from *P. g. glutinosus* (Table 3). The comparatively short DNA sequence phase, with only five published studies to date, is interesting in two respects. First, no new lineages have been proposed. This is likely due to the extensive sampling by Highton and colleagues (Highton 1962, Highton 1989, Highton and Peabody 2000) and the success of allozyme techniques in detecting independent lineages (Highton 2000). Second, although some authors have voiced reservations over the species status of all *P. glutinosus* group members (e.g., Weins et al. 2006), there have been no formally proposed changes to the 28 species recognized by morphology and allozymes (Table 3). Studies published during the DNA sequence phase (2001-present) have focused on addressing evolutionary questions and resolving evolutionary relationships rather than on species delimitation (Weisrock et al. 2005, Kozak and Weins 2006, Kozak et al. 2006, Weisrock and Larson 2006, Wiens et al. 2006).

Species delimitation in the *Plethodon glutinosus* group

Researchers attempting to delimit species in the *Plethodon glutinosus* group have had to contend with the four problems associated with species delimitation discussed above. Systematic research into the group has a long history, beginning with Gilliam (1818) and Green (1818), with researchers using a diverse array of delimitation methods. Species have been delimited, therefore, using many different concepts (e.g., typological, biological, phylogenetic, evolutionary). Furthermore, diversification in the *P. glutinosus* group has occurred in a relatively recent rapid radiation (Highton 1995, Wiens et al. 2006, Kozak et al. 2006) making incomplete lineage sorting and hybridization persistent problems. Lastly, morphological differences among many members are sparse (Highton 1989), creating many cryptic species complexes within the group. These characteristics have led to a long and contentious history of species delimitation that has only recently subsided.

The problem of cryptic species

Before the advent of molecular tools, researchers had to rely solely on limited morphological differences to diagnose species in the *P. glutinosus* group (Wake et al. 1983, Larson 1984, 1989). In the early years of systematics of this group, the task was relatively straightforward as a few characters are reliable in diagnosing species or clusters of species. For example, *P. glutinosus* sensu stricto was initially described because of the presence of dorsal and lateral white flecks (Green 1818), *P. jordani* was distinguished by the presence of red cheek patches (Blatchley 1901), *P. shermani* (except for populations in the Unicoi Mountains) by the presence of red legs (Stejneger 1906), *P. yonahlossee* because of reddish dorsal pigmentation (Dunn 1917), and *P. metcalfi* sensu stricto because it lacked white or red pigmentation of any sort (Brimley 1912). Attempts at finding other diagnostic morphological characters were largely unsuccessful and met with controversy. For example, the presence of brassy flecks on the dorsum of southeastern populations of *P. jordani* complex species were used in the description of *P. clemsonae* by Brimley (1927). The species was subsequently treated as a subspecies of *P. shermani* (Hairston and Pope 1948), a race of *P. metcalfi* (Mittleman 1948), and a subspecies of *P. jordani* (Hairston 1950). It was not until the application of allozymes by Highton and Peabody (2000) that the species was found to be genetically indistinct from *P. metcalfi* and the taxonomy stabilized (see Table 2). As another example, Pope and Hairston (1948) used costal groove counts, extent of dark ventral pigmentation, and number of vomerine teeth to distinguish *P. shermani rabunensis* and *P. s. melaventris* from other members of what they term the "*jordani-metcalfi*" group. Mittleman (1948), however, pointed out that costal groove counts are: (1) variable within a species and (2) dependent upon preservation and development. Therefore, a count of 13 (the number given for the type specimen of *P. s. rabunensis*) may easily be interpreted as 15, which is within the range recorded for *P. clemsonae* and *P. metcalfi*. Mittleman (1948) further points out ventral pigmentation is variable within both *P. metcalfi* and *P. clemsonae*, encompassing descriptions from dark gray to black, the same as given for *P. s. rabunensis* and *P. s. melavaentris*. There is similar overlap in vomerine tooth count (Mittleman 1948).

Morphologically intermediate hybrid animals confused early taxonomists as well. The early detection of *P. shermani* x *P. glutinosus* (now *P. teyahalee*) hybrids caused Bishop (1941) to reclassify *P. shermani* as a subspecies of *P. glutinosus*. The same hybrid morphology caused Hairston (1950) to classify *P. teyahalee* as a subspecies of *P. jordani*. Currently, *P. teyahalee* is generally accepted as a full species under the *P. glutinosus* complex. Interestingly, however, Highton (1989) suggested that *P. teyahalee* might be of hybrid origin, having arisen through one or more hybridization events between *P. shermani* (of the *P. jordani* complex) and *P. cylindraceus* (of the *P. glutinosus* complex). Another controversy surrounding *P. teyahalee* involves the putative hybrid nature of the type specimen. Animals collected from Teyahalee Bald in the Snowbird Mountains of North Carolina were originally named as a subspecies of *P. jordani* (*P. j. teyahalee*) by Hairston (1950). Highton (1983, 1989) suggested the specimen is of hybrid origin but most closely resembles *P. glutinosus*, and consequently elevated the subspecies to full species status. The name *P. teyahalee* was contested by Hairston (1993) since names based on hybrid specimens are invalid under the International Code of Zoological Nomenclature; Hairston (1993) proposed the name *P.*

oconaluftee instead. The name *P. teyahalee* remains prominent in the literature, although some authors adhere to the name *P. oconoluftee* (e.g., Petranka 1998, Dodd 2004).

Later studies of morphological variation have also failed to satisfactorily delimit species boundaries. Highton (1962) considered vomerine tooth count and pigmentation in a genus-wide analysis, and concluded, “Geographic variation in the *Plethodon* is sometimes great, spectacular examples occur. Even so it is necessary to be so arbitrary in the choice of characters used to demarcate subspecies that I fail to see the advantage of their continued use.” Manzo (1988) conducted a morphometric analysis of five species of the *P. jordani* complex (*P. cheoah*, *P. jordani*, *P. metcalfi*, *P. montanus*, and *P. shermani*) using 12 measurements. Only 54.9% of specimens were correctly assigned to their known species (based on allozyme data, morphology, and geography). Moreover, results showed a near complete lack of congruence between phylogenies based on morphometric data versus allozyme data. More recently, Carr (1996) performed the most comprehensive morphological analysis to date. Using 10 characters, the author analyzed 1,022 specimens from 26 populations representing 14 of the 16 species of the *P. glutinosus* complex, as well as *P. petraeus* and *P. yonahlossee*. Results showed that body size explained 75.2% of observed morphological variation, although there were also differences in shape and proportion. Even so, only 61.5% of specimens were classified into their correct species. After controlling for variation in body size, correct classification fell to 50.0%. These findings led the author to conclude that intrapopulational variation is as great as interpopulational variation, and therefore morphology was not useful in species diagnosis.

Species delimitation in the allozyme phase ushered in a new age of *Plethodon* systematics. With the publication of Highton (1989), the number of species recognized within the *P. glutinosus* complex (the largest complex within the group) grew from four to 16 (four of which were formerly considered subspecies within *P. glutinosus*; see Table 2). Highton (1989) used a Nei's genetic distance of 0.15 (based on 22 allozyme loci) as a threshold; populations separated by at least this distance were considered to be sufficiently divergent to warrant species status. Highton justified use of this cutoff based on the following: (1) it is consistent with some morphological characters and (2) previous authors (Baverstock et al. 1977, Thorpe 1982) have suggested using a cutoff of 15% divergence to separate species. In their paper on species concepts and the nature of species, this method was openly criticized by Frost and Hillis (1990) on multiple accounts: (1) a genetic threshold of 0.15, or the use of any genetic threshold, is arbitrary. (2) It is difficult to determine if allopatric taxa are reproductively isolated or not. Therefore, to say that a cutoff of 15% probably makes them different biological species is unwarranted. (3) Highton himself adjusts the cutoff in order to make two exceptions, once to raise a population to species level despite a genetic distance of 0.146 and once to group two populations with a distance of 0.155. (4) The use by Baverstock et al. (1977) of a genetic threshold of 15% does not correspond to a Nei's genetic distance of 0.15. Populations that differ by 15% in the number of fixed loci can actually vary between 0 and infinity, depending upon the level of polymorphism. (5) Since genetic distance is heavily influenced by the level of polymorphism, simply choosing faster or slower evolving genes can lower or raise the resulting genetic distance, thereby making a genetic cutoff even more arbitrary. (6) There was clearly (according to Frost and Hillis 1990 and

Highton 1989) a failure in the clustering algorithm in assigning *P. albagula* to the proper population. As reported by Highton, this species more closely resembled eight other clusters than it did other populations of *P. albagula*. The authors point out that since *P. albagula* did not cluster into a monophyletic group, then other species may not be clustered accurately either (Frost and Hillis 1990).

Highton (1990) defended his use of a genetic cutoff by arguing the following: (1) Use of a genetic threshold of 0.15 is not arbitrary since divergence of this amount successfully unifies geographically contiguous groups that are morphologically and genetically concordant. He further emphasizes that this value is consistent with previously published values for species level divergence (Baverstock et al. 1977 and Thorpe 1982); implying that, rather than arbitrarily adopting divergence values found in other studies using different taxa, his was independent corroborative evidence. (2) His application of a genetic cutoff in delimiting allopatric lineages is necessary since it is impossible to apply the reproductive isolation criteria of the Biological Species Concept to allopatric populations. (3) As a genetic cutoff of 0.15 is only an approximation of the amount of genetic divergence necessary to achieve species status, actual values may differ. It is, therefore, important to consider other lines of evidence such as geography and morphology. (4) Highton amended his statement to read "Two populations that have fixed differences at 15% of their loci would *usually* have…a (Nei's) $D > 0.16$". (5) The presence of polymorphism does not substantially alter the genetic distance as Frost and Hillis (1990) argued. When compared to a corrected genetic distance (denoted D^*), actual values from Hillis's (1985) own work showed only insignificant differences. (6) The last criticism of Frost and Hillis (1990) remained unaddressed. In sum, Frost and Hillis never published a response to Highton's (1990) rebuttal. Although frequently commented on, this public debate seems not to have dissuaded researchers from accepting Highton's taxonomy.

The application of different species concepts

Prior to the early 1970s, no researcher stated explicitly to which species delimitation criterion he or she adhered; however, this period predates the proliferation of species concepts. Since no systematic study published during this time used reproductive isolation criteria, and since morphological characters were the only diagnostic tools at their disposal, researchers during this early period may be said to have used a typological species criteria. This contradicts Highton's (1995) assertion that most *Plethodon* taxonomists since 1945 have used the BSC. In his own work, Highton used morphological characters (pre-1970s) and allozymes (post-1970s) to infer the extent of gene exchange. As the BSC relies chiefly on reproductive isolation, proxies such as the extent of morphological or genetic divergence should, in theory, yield identical species boundaries. Recent studies, however, have empirically demonstrated that applying different species concepts to the same system may yield incongruous results (see above under Difficulties with species delimitation: *Different species concepts*).

Beginning in 1978 with the clumping of *P. longicrus* into *P. yonahlossee* by Guttman et al. (1978), which used allozyme data for the first time, researchers switched from a typological species concept to a version of the PSC or Evolutionary Species Concept. Given the way in which species delimitation was practiced by researchers during this time, there is no distinction between the two, related concepts. This view is shared by Tilley et al. (2008) who briefly summarized the use of species concepts in allozyme studies. These authors claim to use the same methodology as Highton (1989) and Highton and Peabody (2000), and further claim use of the General Lineage Concept (de Quieroz 1998) and elements of the Evolutionary Species Concept (Wiley 1978, see also Frost and Hillis 1990). This contradicts Highton and Peabody (2000) who explicitly state adherence to the BSC, despite the difficulty encountered when applying it to a group which experiences such widespread hybridization.

Highton and Peabody (2000) and Highton (2000) set forth a list of lines of evidence that may be used to delimit species. These authors rely heavily on allozyme data and used similar guidelines in delimiting many of the presently recognized species in the *P. glutinosus* group. The lines of evidence are: (1) a Nei's *D* of 0.15 or greater, (2) morphologically distinct groups, (3) a lack of interbreeding among sympatric lineages, (4) little or no gene flow among parapatric lineages, and (5) patterns of geographic genetic variation within putative species which suggest the presence of gene flow. Furthermore, Highton and Peabody (2000) use two additional lines of evidence to delimit species of the *P. jordani* complex: (1) The Good-Wake method, which regresses the genetic distance versus geographic distance of paired samples (Good and Wake 1992). If the regression line passes through the origin, then no discontinuity exists and it can be inferred that the samples belong to a single interbreeding population. (2) Highton's (1989) method whereby the modality (unimodal vs. multimodal) of distribution of *D*-values is determined for a group of samples. A unimodal distribution is expected if all samples are from a single interbreeding population. When applied to the *P. jordani* complex samples, the authors found strong evidence for the presence of seven independent lineages, which they designate as species.

Weisrock and Larson (2006) expand on the anaylsis of Highton and Peabody (2000) by applying additional statistical analyses to the same allozyme dataset, as well as incorporating mtDNA. In their analysis of the *P. jordani* complex, Weisrock and Larson conclude that all seven lineages identified by Highton and Peabody are, in fact, valid species according to the PSC (as defined by Cracraft 1989) and the General Lineage Concept (GLC), since they appear to have the requisite pattern of ancestry and descent. Currently, species status of all seven lineages is widely accepted.

Distinguishing between incomplete lineage sorting and introgressive hybridization

It is often difficult to distinguish between incomplete lineage sorting and hybridization (see above). In the *P. glutinosus* group, however, it is known that historical (Weisrock et al. 2005, Weisrock and Larson 2006) and present-day hybridization (Hairston et al. 1992, Highton and Peabody 2000) occur. Despite the prevalence of hybridization, the Good-Wake method employed by Highton and Peabody (2000) using 22 allozyme loci shows some genetic isolation among many species, suggesting a lack of widespread introgression. This is confirmed by other studies using different molecular markers. Despite known hybridization of *P. jordani* sensu stricto and *P. cheoah* with other members of the *P. glutinosus* group, Weisrock and Larson (2006) found no evidence of widespread introgression of mtDNA. This is in contrast to the same authors' findings for *P. amplus*, *P. meridianus*, *P. metcalfi* and *P. montanus*. Although these four species are differentiated enough at mtDNA loci to diagnose as separate species under the

PSC and GLC, there is strong haplotype sharing, suggesting periods of gene flow. It should also be noted that neither Highton and Peabody (2000) nor Weisrock and Larson (2006) found fixed genetic differences among the sample of 22 allozyme loci (the same set of loci was used in both studies). Additionally, Weisrock et al. (2005) found extensive mtDNA introgression in *P. shermani* from many other species in the *P. glutinosus* group. Interestingly, shared mtDNA haplotypes occur between some species that otherwise have deeply divergent mtDNA lineages (e.g., *P. shermani* and *P. jordani*). This finding suggests that similarity between species results, not from incomplete lineage sorting, but from introgressive hybridization, likely as a result of past range shifts (Weisrock et al. 2005).

Fine-scale analyses of a few of the myriad hybrid zones occurring between species in the *P. glutinosus* group are another approach that has been used to document past and present gene flow. In a 20-year study of a hybrid zone between *P. shermani* and *P. teyahalee* in the Nantahala Mountians, Hairston et al. (1992) found substantial and increasing introgression. The latter species, the authors conclude, is expanding its range upslope into the range of the former, possibly as a result of cessation of logging activity in the 1930s. As part of the same study (over an 18-year period, rather than 20-year period), the authors failed to detect introgression across a hybrid zone between *P. jordani* and *P. metcalfi* at the interface of the Great Smoky and Balsam Mountains (Hairston et al. 1992). Importantly, however, only a few morphological characters were used in this study. Genetic sampling, especially of mtDNA, might yield different results (Funk and Omland 2003, Weisrock et al. 2005).

A third approach used to detect the presence of gene flow is the use of laboratory crosses. Using this method researchers seek to quantify the extent of reproductive isolation between closely related lineages. Reagan (1992) performed sexual isolation experiments on 12 pairs of populations from five species in the *P. jordani* complex (*P. jordani*, *P. metcalfi*, *P. montanus*, *P. cheoah* and *P. shermani*). Mating trials were scored using two indices of mating success, spermatophore deposition and insemination. Results for the two indices are largely concordant; significant sexual isolation (determined using isolation coefficients comparing homotypic versus heterotypic crosses) was found in 10 out of the 12 pairs when considering spermatophore deposition and nine out of the 12 pairs for insemination. Reagan's (1992) dataset includes two crosses between different isolates of the same species (the Cowee Bald and Blue Ridge isolates of *P. metcalfi* and the Wayah Bald and Standing Indian isolates of *P. shermani*). Crosses from homotypic *P. shermani* populations do not show significant sexual isolation. Crosses from homotypic *P. metcalfi* populations, however, do show significant isolation. This finding is consistent with Weisrock and Larson's (2006) finding of considerable mtDNA haplotype structuring and introgression in *P. metcalfi*. Another interesting finding by Reagan (1992) is that some parapatric populations of *P. teyahalee* and *P. jordani* exhibit sexual isolation from one another, while other populations do not show such a pattern. The reproductive isolation criterion of the BSC is clearly not met (Larson and Chippendale 1993), despite Highton's assertion that these species were diagnosed using the BSC (Highton and Peabody 2000).

Although evidence for introgressive hybridization is strong, there is some evidence that incomplete lineage sorting has also played a role in species delimitation in the *P. glutinosus* group. Highton (1995) proposed that the genus *Plethodon* underwent a rapid radiation about 5 mya; the *P. glutinosus* group became especially species rich during this time. The phylogenetic tree created from allozyme data (see Palmer et al. 2005) shows that, while the *P. glutinosus* group is monophyletic, the same is not true for the *P. jordani* and *P. glutinosus* complexes. This general pattern of polyphyly in the *P. jordani* and *P. glutinosus* complexes is corroborated by Wiens et al. (2006) using a phylogeny which included two mitochondrial and two nuclear genes (Figure 2.1). Although the independent phylogenies share clustering of some groups (e.g., *P. fourchensis*, *P. ouachitae* and *P. caddoensis*), the remaining species do not cluster similarly between the two trees. Furthermore, the timing of diversification as estimated by Wiens et al. (2006) is possibly much older than that reported by Highton (1995). In support of Wiens et al. (2006), an estimate of 8-11 mya was also made Kozak et al. (2006) using mtDNA. Such a recent, rapid radiation has likely resulted in incomplete lineage sorting, and may be responsible for at least some of the difficulty in delimiting species boundaries in the group. Regarding species delimitation, Wiens et al. (2006) state "We acknowledge the possibility that some pairs of species in this group may prove to be conspecific upon further investigation…but we consider it unlikely that the number of species in the *glutinosus* group has been substantially overestimated."

Conclusion

Historically, species delimitation in the *Plethodon glutinosus* group has been fraught with difficulties and controversy. This is largely due to rampant homoplasy (Wake 1991) and morphological stasis (Wake et al. 1983, Highton 1989), which have resulted in few morphologically diagnostic characters. Because of extensive sampling by Highton and colleagues (Highton and MacGregor 1983, Highton 1983, 1989, Highton and Peabody 2000), it is unlikely that many more species will be described in the group. Furthermore, species relationships within the group remain ambiguous, likely because rapid radiation of one or a few ancestral forms roughly 10 mya (Highton 1995, Wiens et al. 2006, Kozak et al. 2006), coupled with extensive introgressive hybridization (e.g., Weisrock et al. 2005, Weisrock and Larson 2006), has led to few genetic differences among species. Recent attempts at identifying relationships have focused on expanded mtDNA phylogenies (Kozak et al. 2006) and multilocus nuclear and mtDNA datasets (Wiens et al. 2006). Despite this recent attention, however, the status of species relationships continues to be dynamic, and it is in this area that more extensive multilocus datasets will prove most beneficial.

In recent years, great strides have been made by systematists in resolving the problems of cryptic species, incomplete lineage sorting and introgressive hybridization. This has been primarily done through the use of multilocus datasets (e.g., Belfiore et al. 2008, Brumfield et al. 2008). There has also been considerable attention recently to develop computer programs (e.g., BEST, Liu and Pearl 2007 and Edwards et al. 2007; BUCKy, Ané et al. 2007) to handle such datasets. Furthermore, genomics-based methods show considerable promise for species delimitation as well. The use of whole genomes (or, at least, greatly expanded DNA sequence datasets) in the construction of phylogenetic trees has become feasible for many model groups (see, for example, Rivera 2007).

The utility of DNA barcoding, especially in understudied groups, has been severely criticized (Rubinoff et al. 2006). Barcoding relies on a short segment (~600 bp) of mtDNA (cytochrome c oxidase subunit 1, CO1). Although not directly tested in the *P. glutinosus* group, it is clear that this method would fail to recover species known from morphology, geography, allozymes, nuclear DNA and more extensive mtDNA sampling. Incongruent mtDNA phylogenies (Kozak et al. 2006, Wiens et al. 2006), extensive mtDNA introgression (Weisrock et al. 2005, Weisrock and Larson 2006, see also Chapter III), and extensive hybridization (e.g., Hairston et al. 1992, Highton and Peabody 2000) support this argument. If applied to the *P. glutinosus* group, barcoding would almost certainly fail to capture the complex history of the organisms. As a result, species identifications using barcoding would be suspect at best.

Figure 2.1. Competing phylogenetic trees of the *Plethodon glutinosus* group from (A) Palmer et al. (2005) constructed using allozymes and (B) Wiens et al. (2006) constructed using mitochondrial and nuclear DNA. Species from the *P. jordani* complex are indicated in bold. Shared monophyletic groups are indicated with boxes.

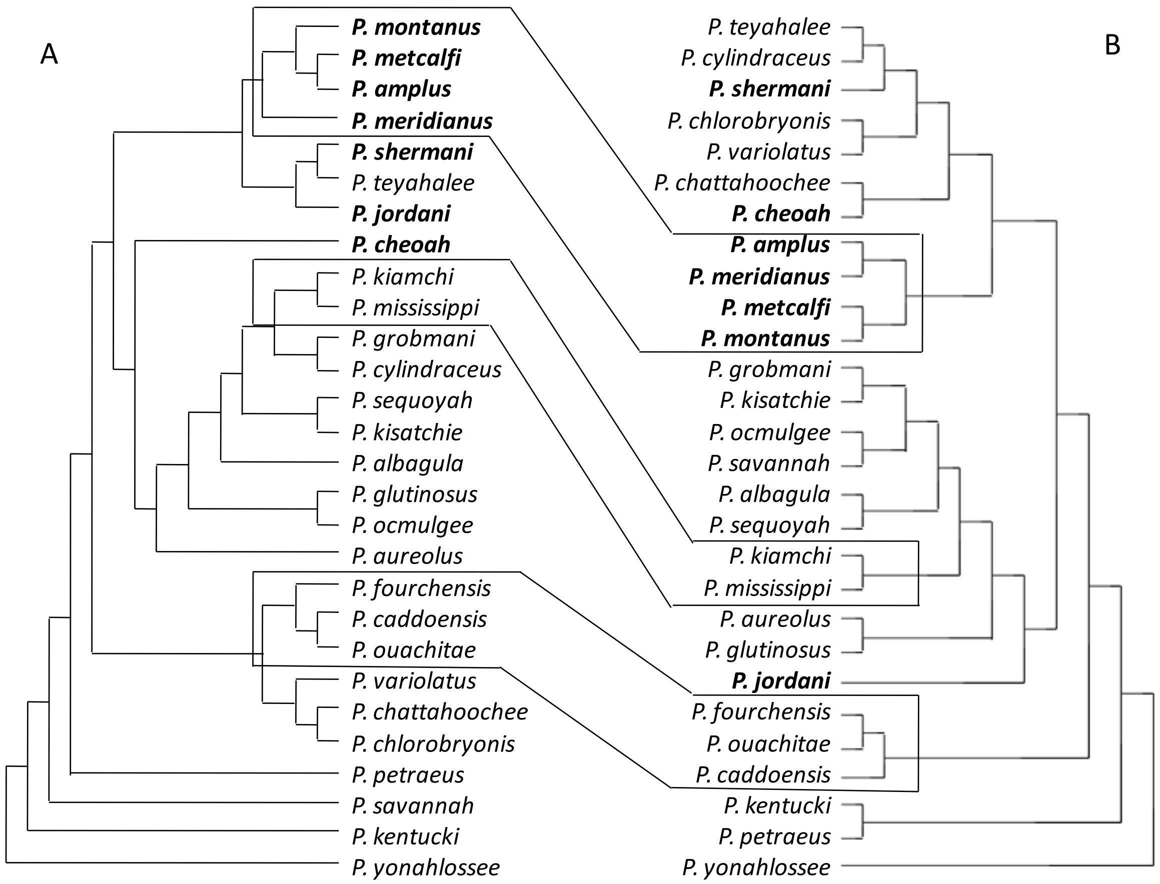
A
P. montanus
P. metcalfi
P. amplus
P. meridianus
P. shermani
P. teyahalee
P. jordani
P. cheoah
P. kiamchi
P. mississippi
P. grobmani
P. cylindraceus
P. sequoyah
P. kisatchie
P. albagula
P. glutinosus
P. ocmulgee
P. aureolus
P. fourchensis
P. caddoensis
P. ouachitae
P. variolatus
P. chattahoochee
P. chlorobryonis
P. petraeus
P. savannah
P. kentucki
P. yonahlossee
B
P. teyahalee
P. cylindraceus
P. shermani
P. chlorobryonis
P. variolatus
P. chattahoochee
P. cheoah
P. amplus
P. meridianus
P. metcalfi
P. montanus
P. grobmani
P. kisatchie
P. ocmulgee
P. savannah
P. albagula
P. sequoyah
P. kiamchi
P. mississippi
P. aureolus
P. glutinosus
P. jordani
P. fourchensis
P. ouachitae
P. caddoensis
P. kentucki
P. petraeus
P. yonahlossee

Table 2.1. Species currently recognized in the *Plethodon glutinosus* complex.

Complex	Species	Complex	Species
P. glutinosus	*P. albagula*	***P. jordani***	*P. amplus*
	P. aureoles		*P. cheoah*
	P. chattahoochee		*P. jordani*
	P. chlorobryonis		*P. meridianus*
	P. cylindraceous		*P. metcalfi*
	P. glutinosus		*P. montanus*
	P. grobmani		*P. shermani*
	P. kiamichi		
	P. kisatchie	***P. ouachitae***	*P. caddoensis*
	P. mississippi		*P. fourchensis*
	P. ocmulgee		*P. ouachitae*
	P. savannah		
	P. sequoyah	**Other species**	*P. kentucki*
	P. teyahalee[*]		*P. petraeus*
	P. variolatus		*P. yonahlossee*

[*] There is disagreement in the literature as to whether the correct name for this species is *P. teyahalee* or *P. oconoluftee*.

Table 2.2. Taxonomic timeline of the *Plethodon glutinosus* group.

Date	Species described	Citation
1818	*P. glutinosus*	Green (1818)
1818	*Salamandra variolata*	Gilliams (1818)
1825	*Salamandra cylindraceus*	Harlan (1825)
1854	*P. variolosum* (changed from *S. variolata*)	Dumeril et al. (1854)
1901	*P. jordani* (red-cheeked form only)	Blatchley (1901)
1906	*P. shermani* (red-legged form only)	Stejneger (1906)
1912	*P. metcalfi*	Brimley (1912)
1917	*P. yonahlossee*	Dunn (1917)
1917	*P. wehrlei**	Fowler and Dunn (1917)
1927	*P. clemsonae*	Brimley (1927)
1933	*P. ouachitae*	Dunn and Heinze (1933)
1941	*P. shermani* considered subspecies of *P. glutinosus*	Bishop (1941)
1944	*P. glutinosus albagula*; two species groups recognized (*P. glutinosus* group: *P. glutinosus glutinosus*, *P. glutinosus albagula*, *P. ouachitae*, *P. wehrlei*, *P. yonalossee*; *P. metcalfi* group: *P. metcalfi*, *P. clemsonae*, *P. jordani*, *P. shermani*)	Grobman (1944)
1948	*P. shermani shermani*, *P. s. rabunensis* and *P. s. melaventris*	Pope and Hairston (1948)
1948	*P. shermani clemsonae*	Hairston and Pope (1948)
1948	*P. shermani rabunensis* and *P. s. melaventris* refuted (all considered *P. metcalfi*)	Mittleman (1948)
1949	*P. dixi*	Pope and Fowler (1949)
1949	*P. glutinosus grobmani*	Allen and Neill (1949)
1950	*P. jordani teyahalee*; *P. jordani* contains seven subspecies (*P. j. jordani*, *P. j. shermani*, *P. j. metcalfi*, *P. j. clemsonae*, *P. j. rabunensis*, *P. j. melaventris* and *P. j. teyahalee*)	Hairston (1950)
1951	*P. caddoensis*	Pope and Pope (1951)
1951	*P. kentucki*	Mittleman (1951)
1953	*P. kentucki* recognized as subspecies of *P. jordani*	Schmidt (1953)
1954	*P. jacksoni*	Newman (1954)
1954	*P. dixi* considered subspecies of *P. wehrlei*; *P. wehrlei* contains two subspecies *P. w. wehrlei* and *P. w. dixi*	Bogert (1954)
1955	*P. kentucki* sunk into *P. glutinosus glutinosus*	Clay et al. (1955)
1956	*P. glutinosus grobmani* refuted	Humphries (1956)

1961	*P. jacksoni* considered subspecies of *P. wehrlei*; *P. wehrlei* contains three subspecies *P. w. wehrlei*, *P. w. dixi* and *P. w. jacksoni*	Cochran (1961)
1962	*P. yonahlossee* group (*P. yonahlossee*, *P. ouachitae*, *P. caddoensis*) and *P. glutinosus* group (*P. jordani*, *P. glutinosus glutinosus*, *P. glutinosus albagula*) recognized	Highton (1962)
1962	*P. longicrus*	Adler and Dennis (1962)
1978	*P. longicrus* sunk into *P. yonahlossee*	Guttman et al. (1978)
1979	*P. fourchensis*	Duncan and Highton (1979)
1979	*P. glutinosus* group recognized (as distinct from *P. wehrlei* group): *P. glutinosus*, *P. jordani*, *P. yonahlossee*, *P. ouachitae*, *P. fourchensis*, *P. caddoensis*	Highton and Larson (1979)
1983	*P. kentucki* resurrected	Highton and MacGregor (1983)
1984	*P. aureolus*	Highton (1984)
1988	*P. petraeus*	Wynn et al. (1988)
1989	*P. albagula* (formerly *P. glutinosus albagula*), *P. chattahoochee*, *P. chlorobryonis* (formerly *P. glutinosus chlorobryonis*), *P. cylandraceus* (formerly *Salamandra cylandracea*), *P. grobmani* (formerly *P. glutinosus grobmani*), *P. kiamichi*, *P. kisatchie*, *P. mississippi*, *P. ocmulgee*, *P. savannah*, *P. sequoyah*, *P. variolatus* (formerly *P. variolosum*)	Highton (1989)
1995	Three complexes and three other species recognized in the *P. glutinosus* group: *P. petraeus*, *P. yonahlossee*, *P. ouachitae* complex (*P. caddoensis*, *P. fourchensis*, *P. ouachitae*), *P. jordani* complex (*P. jordani*), and *P. glutinosus* complex (*P. albagula*, *P. aureolus*, *P. chattahoochee*, *P. chlorobryonis*, *P. cylindraceus*, *P. glutinosus*, *P. grobmani*, *P. kentucki*, *P. kiamichi*, *P. kisatchie*, *P. mississippi*, *P. ocmulgee*, *P. savannah*, *P. sequoyah*, *P. teyahalee*, *P. variolatus*)	Highton (1995)
2000	*P. amplus*, *P. cheoah*, *P. meridianus* and *P. montanus*	Highton and Peabody (2000)
2006	Three clades and five other species recognized: “Clade B”(*P. oconoluftee* and *P. teyahalee* [unsure of species	

status so both included in analysis], *P. cylindraceus*, *P. shermani*, *P. chlorobryonis*, *P. variolatus*, *P. chattahoochee*, *P. cheoah*, *P. amplus*, *P. meridianus*, *P. metcalfi*, *P. montanus*), “Clade A” (*P. grobmani*, *P. kisatchie*, *P. ocmulgee*, *P. savannah*, *P. albagula*, *P. sequoyah*, *P. kiamichi*, *P. mississippi*, *P. aureoles*, *P. glutinosus*), *P. ouchitae* complex (*P. fourchensis*, *P. ouachitae*, *P. caddoensis*), *P. jordani*, *P. kentucki*, *P. petraeus*, *P. longicrus* and *P. yonahlossee* [unsure of species status so both included in analysis]	Wiens et al. (2006)

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[*] *Plethodon wehrlei* is currently recognized as belonging to either the *P. welleri* group (see Palmer et al. 2005) or the *P. welleri-P. wehrlei* group (Wiens et al. 2006). Some early researchers (e.g., Grobman 1944), however, included *P. wehrlei* as a part of the *P. glutinosus* group, and the species is therefore included in this table.

Table 2.3. Phases of species delimitation in the *Plethodon glutinosus* group, the dates during which the phases lasted, and the number of studies published with a focus on taxonomy or systematics.

Phase	Criteria	Dates of Utilization	N (Studies)	# of Species/ Subspecies Described	# of Name Changes*
1	Morphology	1818-1962	29	20	15
2	Allozymes	1978-2000	9	20	2
3	DNA sequence	2001-present	5	0	0

* Indicates the number of times a species or subspecies was clumped into a different species or subspecies, or a previously recognized species or subspecies was resurrected.

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CHAPTER III

Differential introgression of mtDNA relative to nuclear DNA and morphology in a salamander hybrid zone involving three species (Plethodontidae: *Plethodon*)

Abstract

The role of hybridization as an important mechanism in animal evolution is gaining acceptance. Presented here are analyses from a hybrid zone that occurs among the salamanders *Plethodon jordani*, *P. metcalfi*, and *P. teyahalee* from the southern Appalachian Mountains. Spatial patterns of variation are examined at four markers: single nucleotide polymorphisms (SNPs) located in the mitochondrial DNA (mtDNA) ND2 gene and the nuclear DNA ILF3 gene and the morphological markers of red cheek pigmentation and white flecks. Four transects were established: two along high elevation ridgelines predominately connecting the ranges of *P. jordani* and *P. metcalfi*, and two elevational transects connecting hybrid populations of the former species with that of *P. teyahalee*. Localities along these transects were sampled at a fine-scale. Concordance among nuclear DNA and both morphological markers across the four transects is observed. In three of the four transects, however, the pattern of mtDNA is discordant from all other markers, with an overrepresentation of *P. metcalfi* mtDNA in the northern and lower elevation localities than is expected given nuclear DNA and morphology. This finding, in addition to studies demonstrating mating asymmetry and differing

ecological niches, suggests the hybrid zone may be moving south into the range of *P. metcalfi* and downslope into the range or *P. teyahalee*.

Introduction

The importance of hybridization in evolution has long been known to botanists (see Arnold 2006 and Arnold and Meyer 2006), but its importance in zoology has been less clear (Mayr 1963, Coyne and Orr 2004). Seeds of the idea that hybrids are evolutionary dead ends can be seen in Darwin (1859), when he stated that hybrids were nothing more than imperfect offspring of “pure species”. Mayr (1963) voiced a similar sentiment when he wrote, “The total weight of the available evidence contradicts the assumption that hybridization plays a major evolutionary role among higher animals.” Recent studies highlighting the prevalence of hybridization in animals (Mallet 2005) and considerable work demonstrating the porous nature of some species boundaries (Martinsen et al. 2001, Shaw and Danley 2003, Bull et al. 2006, Minder and Widmer 2008) has led researchers to suggest that introgressive hybridization plays an important role in animal, as well plant, evolution (Grant et al. 2005, Arnold 2006, Arnold and Meyer 2006).

Hybrid zones, which form after secondary contact of two partially reproductively isolated populations, have been especially useful in elucidating evolutionary processes. Harrison (1990) viewed these regions of parapatry as “windows on the evolutionary process”, and numerous studies of naturally occurring hybrid zones have yielded considerable insight into the process of speciation (e.g., Szymura and Barton 1991, Tucker et al. 1992, Rieseberg et al. 1999, Vines et al. 2003, Payseur et al. 2004, Teeter et al. 2008). Additionally, because hybrid zones occur between two partially reproductively isolated populations, analysis may uncover the mechanisms maintaining species boundaries (Piálek and Barton 1997, Porter et al. 1997, Kim and Rieseberg 1999, Brumfield et al. 2001, McDonald et al. 2001, Martin et al. 2006). Theoretical work suggests traits or alleles that are under positive selection can introgress across a species boundary even if the remaining genetic background is selected against (Barton 2001). Importantly, introgression can happen as long as the trait or allele is not as disadvantageous in the hybrid background as it is in one of the parental types.

Recent work on hybrid zone movement challenges early assumptions of hybrid zone stasis. In a recent review, Buggs et al. (2007) identified 23 studies which documented hybrid zone movement and another 16 studies which had patterns consistent with movement. These studies utilize two different approaches: First, long-term monitoring of molecular and morphological markers across a hybrid zone is a fool proof method for detecting movement. Second, analyzing differential patterns of introgression at a single point in time may allow for inference of movement. The eventual elimination of the recipient species is one possible outcome of hybrid zone movement. Genetic swamping, the abundant and asymmetrical spread of alleles across species boundaries, results in the extirpation of the recipient species (Rhymer and Simberloff 1996, Wolf et al. 2001, Ayres et al. 2004, Fitzpatrick and Shaffer 2007).

One species rich group where hybridization is especially common is the *Plethodon glutinosus* group (Caudata: Plethodontidae), which is found throughout the southeastern United States. Based on morphology and allozyme data, 28 lineages are currently recognized as species (Highton 1989, Highton and Peabody 2000). Among 17 pairs of sympatric species in the group, five are known to hybridize; and among 16 parapatric pairs, 11 are known to form hybrid zones (Highton and Peabody 2000). Furthermore, it appears that much of this hybridization is introgressive. In a study of a hybrid zone between *P. shermani* and *P. teyahalee*, Hairston et al. (1992) discovered what appears to be massive morphological introgression of *P. teyahalee* into *P. shermani* populations. This hybrid zone movement, the authors conclude, is likely due to the cessation of logging activity early last century. In another example, using mtDNA and allozyme data involving the seven members of the *P. jordani* complex (one complex of many in the *P. glutinosus* group), Weisrock and Larson (2006) found evidence for substantial mtDNA introgression. In a finer scale analysis, Weisrock et al. (2005) uncovered substantial introgression of *P. teyahalee* mtDNA in one geographic isolate of *P. shermani*. Evidence of past genetic swamping by *P. teyahalee* is seen in another isolate of *P. shermani* as well (Highton 1970, 1972, 1989). Evidence for such pervasive introgression supports the idea that hybridization plays an important role in the evolution of salamanders in the *P. glutinosus* group.

A series of hybrid zones among three species in the *P. glutinosus* group occur at the interface of the Great Smoky and Balsam Mountains in the southern Appalachians. The high elevation species *P. jordani* and *P. metcalfi* are known to hybridize along two ridgelines, Balsam Mountain and Hyatt Ridge (Hairston 1950, Highton 1970). These ridgelines are high elevation corridors that connect the Great Smoky to the Balsam Mountains, the ranges of *P. jordani* and *P. metcalfi*, respectively. A third species, *P. teyahalee*, inhabits lower elevations throughout much of the southern Appalachians and hybridizes with the former species at intermediate elevations (Peabody 1978, Manzo

1988, Reagan 1992). The most complete study in this system was done by Hairston et al. (1992). These authors sampled salamanders from the same five localities along the southern portion of Balsam Mountain each year for an 18-year period and recorded the amount of red cheek pigmentation, which is diagnostic for *P. jordani* but absent in *P. metcalfi*. This study documented the extensive nature of hybridization along the ridgeline and, since no movement was detected, yielded some insight on the short-term stability of the hybrid zone.

The existence of hybrid populations among species in the *P. glutinosus* group as documented by previous researchers allows for the opportunity to study the dynamics of naturally occurring hybrid zones. The analyses presented here expand on earlier studies in three ways: (1) patterns of spatial variation are examined for mtDNA, nuclear DNA, and morphological markers; (2) hybrid populations involving *P. jordani*, *P. metcalfi*, and *P. teyahalee* are analyzed across four transects, two along high elevation ridgelines predominately connecting the ranges of *P. jordani* and *P. metcalfi*, and two elevational transects connecting hybrid populations of the former species with that of *P. teyahalee*; and (3) sampling in this study was performed at a spatially fine scale, allowing for increased resolution in the detection of introgression. Results presented here show a pattern of differential introgression of mtDNA relative to nuclear DNA and morphology.

Methods

Sampling

Two high elevation transects were established along Balsam Mountain and Hyatt Ridge. Salamanders were collected from 24 localities along the 24-km long Balsam

Mountain. At each collection locality, a minimum of five salamanders were sampled (Figure 3.1 and Appendix 3.1). Salamanders were collected from 13 localities along 6 km of the southernmost portion of Hyatt Ridge, with a minimum of 10 salamanders sampled per locality (Figure 3.1 and Appendix 3.1). Two elevational transects were also created that connect high and low elevation populations. The Palmer Creek transect begins at 1,390 m in elevation and extends 8 km to 951 m. Salamanders were collected from eight localities, with a minimum of five salamanders per locality (Figure 3.1 and Appendix 3.1). The Mt Sterling transect follows Mt Sterling Ridge for 1.5 km to Mt Sterling summit (at 1,768 m elevation) before descending for 2 km to 1,134 m. Salamanders were collected from 13 localities, with a minimum of 5 salamanders sampled at each locality (Figure 3.1 and Appendix 3.1).

Parental animals from each of the three species were also sampled at locations distant from known areas of hybridization (Figure 3.1 and Appendix 3.2). These included 8 localities in the Great Smoky Mountains (the range of *P. jordani*), 6 from the Balsam Mountains (within the range of *P. metcalfi*), and 6 from low elevations in the Great Smoky Mountains (within the range of *P. teyahalee*). It should be noted that Highton and Peabody (2000) and Weisrock and Larson (2006) uncovered two genetically divergent lineages of *P. metcalfi* corresponding to the Balsam and Blue Ridge Mountains. As only populations from the Balsam Mountains are known to hybridize with *P. jordani*, only these populations of *P. metcalfi* were sampled.

Tissue collection and DNA extraction

Samples were collected in the field from 2004 to 2007 during the months of May to July. Salamanders were captured by hand and 10-20 mm of the tail tip removed for genetic analysis. In the field, vials containing tissue samples were immediately placed in an ice-salt mixture (approximately -20^{o}C) until they could be transferred to liquid nitrogen 3-48 hours later. At the end of the field season, samples were removed from the liquid nitrogen and stored at -80^{o}C. All samples have been cataloged into the tissue collection of the Division of Reptiles and Amphibians, Museum of Zoology at the University of Michigan (under accession number 2008-09 no. 3, and uniquely identified by field number; Appendices 3.3 and 3.4).

DNA was extracted from tail tissue using a standard phenol-chloroform protocol (Museum of Vertebrate Zoology, University of California, Berkeley, CA). Briefly, 0.5-20 µg of frozen tissue was washed in 1 ml of cold STE buffer, and then incubated in a mixture of lysis buffer, proteinase K, and RNase A. Samples were centrifuged and pellets discarded. The resultant supernatant was subjected to three rounds of purification with a phenol-chloroform mixture and centrifugation. DNA was precipitated in ~900 ml of cold 95% ethanol, centrifuged, and the supernatant discarded. The resultant pellet was washed twice with 70% ethanol, allowed to dry, and resuspended in 100 µl of TE buffer.

Morphological markers

Animals were scored in the field for two morphological markers. The first, red cheek pigmentation, is diagnostic for animals captured within the range of *P. jordani*, and is absent in parental populations of *P. metcalfi* and *P. teyahalee*. Animals were scored on a 14-point scale, with 0 indicating the complete absence of red cheek pigmentation and 13 indicating bright and pervasive red cheek pigmentation, extending onto the throat, shoulders, and forelimbs. Assigned scores accounted for both extent and intensity of red pigmentation, as well as both the right and left cheeks. The second marker, white flecking, is diagnostic of pure *P. teyahalee*, and is absent in parental populations of *P. jordani* and *P. metcalfi*. The amount and pattern of white flecking is variable in *P. teyahalee*, but lateral flecks are generally abundant and large, while dorsal flecks are sparse and small. White flecks were scored as being either present or absent. In order to achieve consistency in scoring the morphological markers, all animals were scored by MWHC. Digital photographs of the right and left sides of the head were taken of each animal. These images have been cataloged into the digital image collection of the Division of Reptiles and Amphibians, Museum of Zoology at the University of Michigan (under accession number 2008-09 no. 3, image numbers 49-960, and uniquely identified by field number; Appendices 3.3 and 3.4).

mtDNA marker

A series of two single nucleotide polymorphisms (SNPs) were identified in the mtDNA gene NADH subunit II (ND2): the first distinguishes *P. jordani* from *P. metcalfi* and *P. teyahalee*, and the second distinguishes *P. teyahalee* from *P. jordani* and *P. metcalfi*. Thus, when used in tandem, the SNPs were diagnostic for each species. The panel used to identify these single nucleotide differences consisted of the following pure parental samples: 18 animals from 8 populations within the range of *P. jordani*, 16 animals from 5 populations within the range of *P. metcalfi*, and 13 animals from 6 populations within the range of *P. teyahalee* (Figure 3.1 and Appendix 3.3). Approximately 950 base pairs of the ND2 gene, the entire tRNA$^{\text{Ala}}$, and a portion of the tRNA$^{\text{Trp}}$ gene were amplified with the forward primer MC001 (5’-TTTCTAACCCAATCTATAGCATCC-3’) and the reverse primer MC002 (5’-GTCTTGCAAGTTCGAGTCAGA-3’), designed using the on-line software Primer3 (Figure 3.2A). Polymerase chain reaction (PCR) protocols followed Weisrock et al. (2001), with the inclusion of a 5 min hot start at 95°C and a 7 min final extension at 72°C. Sequencing was performed on an ABI 3730 XL automated DNA sequencer through the University of Michigan DNA Sequencing Core facility. Resulting sequences were aligned using Sequencher version 4.8.

Scoring samples at the mtDNA marker was done using restriction fragment length polymorphism (RFLP) digests. The PCR product for each sample was divided into two equal aliquots and digested with *BanI* and *MfeI* restriction enzymes. When used in tandem, RFLP digestions were unambiguous when scoring at the ND2 locus. Digestion with *BanI* cut the PCR product of *P. jordani* into fragments with approximate lengths of 300 and 650 base pairs, while leaving the products of *P. metcalfi* and *P. teyahalee* whole. Similarly, digestion with *MfeI* cut the PCR product of *P. teyahalee* into fragments with approximate lengths of 280 and 670 base pairs, while leaving the products of *P. jordani* and *P. metcalfi* whole (Figure 3.2A). Because of a polymorphism in *P. jordani*, products for this species were occasionally cut with *MfeI*; however, since *P. teyahalee* was never cut with *BanI*, resulting fragments remained diagnostic. Banding patterns from the RFLP digestions were visualized and scored on 2% NuSieve gels.

Nuclear DNA marker

As with the mtDNA marker, a panel was developed to identify diagnostic nuclear SNPs. The panel consisted of 12 animals from 6 localities in the range of *P. jordani*, 11 animals from 5 localities in the range of *P. metcalfi*, and 8 animals from 5 localities in the range of *P. teyahalee* (Figure 3.1 and Appendix 3.3). As in the mtDNA analysis, two SNPs were identified that, when used in tandem, could distinguish the three parental species. No restriction enzymes with suitable recognition sites could be found. Therefore, scoring samples at the nuclear DNA marker were done by sequencing each PCR product and scoring the sequences by eye. Approximately 280 base pairs of the middle exon (and partial sequences of the surrounding introns) in the nuclear gene interleukin enhancer binding factor 3 (ILF3) were amplified with the forward primer MC003 (5'-CCAGGCATTTATGCATCCTT-3') and the reverse primer MC004 (5'-CGTGCTAGCCTCGGTAACAT-3'), designed using Oligo version 6.71 (Figure 2B). PCR was performed using a hot start of 94^{o}C for 3 min, 20 cycles at 94^{o}C for 30 sec, 65^{o}C minus 0.5^{o}C/cycle for 30 sec, and 72^{o}C for 1 min, followed by 20 cycles at 94^{o}C for 30 sec, 55^{o}C for 30 sec, and 72^{o}C for 1 min, with a final extension at 72^{o}C for 8 min (E. Jockusch pers. comm.). Sequencing and alignments were performed as described above.

Hybrid zone analysis

Two different types of analyses were performed in order to explore differential patterns of introgression. The first consisted of two chi-square contingency table tests on the entire dataset that were used specifically to test for differential introgression of mtDNA relative to nuclear DNA. The first test was performed on individual samples and tested the null hypothesis that nuclear and mtDNA haplotypes were over- or underrepresented. The second test compared sample localities that were classified by their most common haplotype at the nuclear and mtDNA markers and tested the null hypothesis that *P. jordani*, *P. metcalfi*, and *P. teyahalee* haplotypes are equally well represented among the collection localities. In both tests, significance was assigned at the $P \leq 0.05$ level.

Maximum likelihood was used to compare the relative abundance of nuclear and mtDNA genotypes across the entire dataset. Like the contingency table tests, this analysis was used to assess coincidence among molecular markers; however, multimodel testing allowed different genotypic combinations to vary independently. Samples were grouped according to whether they were: (1) homozygous at the nuclear marker and had homospecific mtDNA, (2) homozygous at the nuclear marker and had heterospecific mtDNA, (3) heterozygous at the nuclear marker and had mtDNA from one of the nuclear parents, or (4) heterozygous at the nuclear marker and had mtDNA from a third parental species. Generalized linear models (GLMs) were then created to compare the likelihood that the multilocus genotype frequencies could be predicted by the single-locus genotype frequencies. Eleven different models were tested: an independence (null) model in which each possible genotypic category is predicted independently by the frequency of the single-locus genotypes, a symmetrical model in which each of the four classes is predicted independently from one another, and nine asymmetrical models in which each of the six nuclear genotypes (three homozygous and three heterozygous combinations) and each of the three mtDNA genotypes is predicted independently from the other multilocus genotypes.

Two methods were used to assess the suitability of each model. The first is the Akaike information criterion (AIC). Rather than finding the single best model, the AIC is a multimodel inference tool that assesses the suitability of the full set of models given the data (Burnham and Anderson 2004). Models are ranked hierarchically according to their AIC values, with lower values indicating higher suitability. Often, as is done here, AIC values are given as ΔAIC, which is the difference of each AIC value from that of the best model. The second method uses the residual deviance as a measure of the goodness-of-fit of a model relative to a saturated model, which acts as a baseline (Agresti 2007). Higher values indicate more variation is unaccounted for by the model and, therefore, lower values indicate models with a better fit. Significance levels are determined by comparison of each model to the saturated model. Therefore, an insignificant residual deviance ($P > 0.05$) means the residual variance is adequately explained as sampling error and the model is an adequate description of the data. All analyses were performed using R version 2.4.1.

Ecological niche modeling

Ecological niche models were created for *P. jordani* and *P. metcalfi*. Georeferenced localities for *P. jordani* (n = 374) and *P. metcalfi* (n = 289) are a subset of those used by Kozak and Wiens (2006) and were obtained by those authors from the collection of the U. S. National Museum. Most specimens used in the analysis were collected by R. Highton and assigned to species using morphology and allozymes. Any specimen with uncertain identification was omitted from the analysis (Kozak and Wiens 2006). Models for *P. teyahalee* are not included in the hybrid zone analysis, as the model resolution is not fine enough to permit meaningful interpretation in the narrow area of hybridization.

Models were created using Maxent version 3.2.1 (Phillips et al. 2004, Phillips et al. 2006). Maxent is a machine-learning method which utilizes presence-only data in the construction of models of species distributions. Like other niche modeling methods, the objective of Maxent is to predict environmental suitability within a specified area given the environmental conditions of known collection localities of a population or species of interest. Maxent does this by creating a probability distribution of habitat suitability for all grid cells in the specified region by computing the probability distribution of maximum entropy, i.e., a distribution that is closest to uniform with respect to the average of environmental conditions at the known collection localities. Habitat suitability values for each grid cell are given as a probability, and probability values for all grid cells must sum to 1.0. Each cumulative probability value is subsequently multiplied by 100 to give a percentage. Raster files of habitat suitability values created in Maxent were converted to ASCII files and viewed in ArcGIS version 9.3.

The bioclimatic variables used to model species distributions consisted of the 19 available from the WorldClim database (www.worldclim.org; Table 3.1). Many previous ecological niche modeling studies attempted to minimize non-independence among variables by including representatives from groups of highly correlated variables. Maxent differs from many other niche modeling programs in that it assesses and incorporates variables and the interactions between variables, thus accounting for non-independence (Phillips et al. 2006).

Results

Every salamander captured within the range of pure *Plethodon jordani* had at least some red cheek pigmentation (mean = 8.02, range = 1-13, n = 90), while red pigmentation was entirely absent in populations of pure *P. metcalfi* and *P. teyahalee*. Similarly, the presence of white flecks was found to be diagnostic for pure *P. teyahalee* (n = 11) and was completely absent in populations of pure *P. jordani* and *P. metcalfi* (Appendix 3.3). Within the hybrid zone, the number of samples collected per transect are as follows: Balsam Mountain, 5-16 per locality, 264 samples total; Hyatt Ridge, 10-15 per locality, 155 samples total; Palmer Creek, 5-16 samples, 86 samples total; and Mt Sterling, 5-13 per locality, 140 samples total (Appendix 4). Salamanders captured within the hybrid zone show a wide range of cheek pigmentation scores (range = 0-13, n = 740), and 14 individuals showed the presence of both red cheek pigmentation and white flecks.

Most combinations of genotypic classes are represented in the hybrid zone (Table 3.2). Curiously, no *P. metcalfi-P. teyahalee* heterozygotes were found at the nuclear marker. Similarly, no individuals were found that were homozygous for *P. teyahalee* at the nuclear marker while having *P. jordani* mtDNA. Other genotypic classes are uncommon. For example, only a single individual was sampled that was heterozygous for *P. jordani* and *P. metcalfi* at the nuclear marker but had *P. teyahalee* mtDNA. Similarly, only two individuals were sampled that were homozygous for the *P. metcalfi* nuclear allele but had *P. teyahalee* mtDNA. Most other hybrid genotype combinations are moderately well represented. For example, *P. jordani-P. metcalfi* heterozygotes at the nuclear marker with *P. jordani* mtDNA were found in 28 samples; *P. jordani-P. teyahalee* heterozygotes at the nuclear marker with *P. metcalfi* mtDNA were found in 15 samples; and *P. jordani-P. teyahalee* heterozygotes with *P. jordani* mtDNA were found in 8 samples. Results from the contingency table test on individual samples show that *P. metcalfi* mtDNA is most common, while *P. jordani* nuclear DNA is most common (χ^2 = 193.1835, D.F. = 2, P < 0.0001; Table 3.3). Similarly, results from the contingency table test on sample localities show that *P. metcalfi* mtDNA is most common and *P. jordani* nuclear DNA is most common at the majority of localities (χ^2 = 9.0195, D.F. = 2, P = 0.0110; Table 3.4). This suggests *P. metcalfi* mtDNA is more widespread than *P. metcalfi* nuclear DNA and *P. jordani* nuclear DNA is more widespread that *P. jordani* mtDNA.

The general linear models depict general patterns of association that might be functions of spatial structure, nonrandom mating, natural selection, or any combination thereof. Based on resulting ΔAIC values and residual deviances, the models may be categorized into three groups (Table 3.5). The first group contains those models with large ΔAIC values and significant residual deviances, meaning these models are not suitable given the data. This group includes the null model, the symmetrical model, and the following asymmetrical models: *P. metcalfi* / *P. teyahalee* heterozygotes, *P. metcalfi* homozygotes, *P. jordani* / *P. metcalfi* heterozygotes, and *P. jordani* / *P. teyahalee* heterozygotes (all are nuclear DNA genotypes). The second group contains those models with moderate ΔAIC values and smaller, although still significant, residual deviances. This group includes: *P. jordani* homozygotes (nuclear DNA genotype), and the models varying *P. jordani* and *P. metcalfi* mtDNA. The third group contains two models which have low ΔAIC values and insignificant residual deviances. These models – *P. teyahalee* homozygotes (nuclear DNA genotype) and *P. teyahalee* mtDNA – are the only two models not rejected by the goodness-of-fit test. Overall, these results demonstrate associations among genotypes, with some genotypic combinations being under- or overrepresented.

Three patterns emerge from the Balsam Mountain transect: First, at four localities near the center of the transect (LB, LC, PO, and FR), the presence of *P. teyahalee* was detected both morphologically and genetically (Figure 3.3). This occurs at Pin Oak Gap, a low point in the otherwise high elevation of the Balsam Mountain ridgeline. Second, frequencies of the *P. jordani* nuclear marker and the incidence of red cheek pigmentation are in close agreement (Figure 3.3). That is, populations from localities along the ridgeline that show a predominance of the *P. jordani* nuclear marker also have a high cheek pigmentation score (e.g., localities LG, BH, BG, and SP). Third, frequencies of *P. jordani* mtDNA are largely discordant with respect to those of the nuclear marker and red cheek pigmentation. This is most clearly seen in localities GF, BI, LG, BG, LB, SM, SP, HC, CB, and CA (Figure 3.3).

The pattern of differential introgression is not seen in the Hyatt Ridge transect (Figure 3.4). Rather, there is coincidence among all markers. Also unlike Balsam Mountain, *P. teyahalee* markers do not appear on Hyatt Ridge, except for one individual that is heterozygous at the nuclear DNA marker (with *P. jordani* mtDNA and no white flecks) and one apparently pure *P. teyahalee* from the southernmost collection locality (which has a lower elevation than most other sites on the ridgeline).

The pattern of differential introgression is strongly apparent in the Palmer Creek transect (Figure 3.5). The upslope (western) end of the transect begins in a hybrid population of *P. jordani* and *P. metcalfi* (locality PC); however, nuclear and mtDNA markers show a prevalence of *P. metcalfi* alleles. The average cheek pigmentation score is 0.9, with 2 out of 11 animals having some red pigmentation, and a considerable amount of *P. jordani* nuclear DNA (8 out of 20 allele copies). There is, however, a complete absence of *P. jordani* mtDNA at this site. The presence of the *P. jordani* nuclear marker extends downslope to 1000 m elevation, almost to the valley floor. The presence of some red cheek pigmentation extends to the lowest elevation sampled (locality BK at 951 m with an average cheek pigmentation score of 0.125). This pattern is in contrast to the complete absence of *P. jordani* mtDNA along the transect. Patterns in white flecks are consistent with those of red cheek pigmentation and nuclear DNA.

Patterns of marker frequencies are more complicated along the Mt Sterling transect (Figure 3.6). Along the ridgeline of the western portion of the transect (localities ZI-ZM; 1,561-1,768 m elevation) there is a predominance of *P. jordani* nuclear DNA, mtDNA, and morphology, although *P. metcalfi* mtDNA may be slightly overrepresented. At localities beginning immediately off the ridge top, there is a complete absence of *P. jordani* mtDNA. This is in sharp contrast to the patterns of the *P. jordani* nuclear marker and red cheek pigmentation, both of which predominate to an elevation of 1,280 m. As in the Palmer Creek and Balsam Mountain transects, patterns in *P. teyahalee* (white flecks) are largely coincident with those of red cheek pigmentation and nuclear DNA.

Ecological niche modeling results are presented in Figures 3.3 and 3.7. The models show higher predicted suitability values for *P. jordani* in comparison to *P. metcalfi* over much, but not all, of the hybrid zone. The values for *P. jordani* are high (80-100%) throughout the northern areas of the hybrid zone, and moderately high (60-80%) in the southern areas. The distribution of highly suitable habitat for *P. metcalfi* is patchy, concentrated in the southern areas of the hybrid zone, and is often restricted from the highest elevations. When suitability values within grid cells are compared between the two species at the transect collection localities, *P. jordani* is seen to have higher predicted suitability than *P. metcalfi* at all localities except the southernmost sites (PG, ST, WK, and HT; Figure 3.3).

Out of the 19 bioclimatic variables used to construct the ecological niche models, two highly correlated variables emerged as important (Table 3.1). BIO14, precipitation in the driest month, and BIO17, precipitation in the driest quarter, together explain 77.4% of the predicted distribution for *P. jordani* and 84.7% for *P. metcalfi*. A third variable, precipitation seasonality (BIO15), explains a further 6.9% of the distribution of *P. jordani* and 7.9% of the distribution for *P. metcalfi*.

Discussion

When samples from all four transects are combined, a clear pattern of differential introgression of nuclear and mtDNA emerges. This is most clearly seen in the contingency table results, which show a significant overrepresentation of *Plethodon metcalfi* mtDNA and *P. jordani* nuclear DNA. Results from the general linear models reflect more complex patterns of species interactions. The best fitting models are those in which *P. teyahalee* homozygotes at the nuclear marker or *P. teyahalee* mtDNA vary in abundance. Specifically, the pure *P. teyahalee* genotype is overrepresented, which suggests *P. teyahalee* is less likely to form hybrids than are *P. jordani* and *P. metcalfi*. This finding is consistent with observations that *P. teyahalee* is the most ecologically (based on elevation differences in species ranges) and morphologically (Highton and Peabody 2000) divergent of the three species. The group of models with moderate ΔAIC values show interesting trends, although the results are not significant (i.e., residual deviances have P-values < 0.05). One trend observed in the general linear model results is the overrepresentation of *P. jordani* homozygotes at the nuclear marker, which suggests *P. jordani* hybrids are more likely to backcross with *P. jordani* than with *P. metcalfi*. Another trend is the atypical patterns of *P. jordani* and *P. metcalfi* mtDNA, which reflects an overrepresentation of *P. metcalfi* mtDNA and a subsequent underrepresentation of *P. jordani* mtDNA.

The pattern of differential introgression that is observed in the combined dataset is clearly seen in three of the four individual transects: Balsam Mountain, Palmer Creek, and Mt Sterling. A second pattern that emerges is the general concordance of nuclear DNA and morphology across all four transects. In the Balsam Mountain transect, where hybridization occurs predominately between *P. jordani* and *P. metcalfi*, there is a shift of *P. metcalfi* mtDNA northwards relative to *P. jordani* nuclear DNA and morphology (Figure 3.3). Interestingly, this pattern is not seen in the Hyatt Ridge transect (Figure 3.4). One possible reason for this discrepancy is that Hyatt Ridge, while extending north into the range of *P. jordani*, does not extend into the range of pure *P. metcalfi* in the south. Rather, the ridge descends into a valley, i.e., into habitat that is unsuitable for *P. metcalfi* and *P. jordani*. Thus, while gene flow from pure populations of *P. jordani* may occur from the north, there is currently no such gene flow from pure *P. metcalfi* in the south. In the Palmer Creek and Mt Sterling transects there is a clear discordance, with *P. jordani* mtDNA being restricted to the highest elevations sampled despite the presence of *P. jordani* nuclear DNA and morphology extending much of the way to the valley below (Figures 3.5 and 3.6).

Numerous studies have documented differential introgression of mtDNA relative to nuclear DNA and morphology (Funk and Omland 2003, Chan and Levin 2005). Many of these are phylogenetic studies between closely related species that share select mtDNA haplotypes, suggesting a pattern of current or historic mtDNA gene flow (e.g., Weisrock and Larson 2006, Linnen and Farrell 2007). Many other cases documenting mtDNA introgression come from studies of naturally occurring hybrid zones showing shifts in clines of mtDNA relative to nuclear DNA and morphology (e.g., Arntzen and Wallis 1991, Sequeira et al. 2005, Vörös et al. 2006, Brito 2007, Hofman and Szymura 2007, Leaché and Cole 2007, Kawakami et al. 2008). Natural selection has been proposed as a source of discordance between mtDNA and nuclear markers in some systems (e.g., Doiron et al. 2002, Bachtrog et al. 2006). In this system, positive selection acting on *P. metcalfi* mtDNA would yield a pattern of differential introgression. This hypothesis is difficult to test, and selection remains a possible cause of discordance in this system.

Another hypothesis that accounts for the differential introgression seen in the Balsam Mountain transect is that the hybrid zone is moving southward into the range of *P. metcalfi*. Since mtDNA is maternally inherited, under this scenario, the absence of *P. jordani* mtDNA from the expanding front may be a result of mating asymmetry as found by Reagan (1992). That study used laboratory crosses and measured two different proxies of mating success, spermatophore deposition and insemination. Heterospecific crosses between female *P. metcalfi* and male *P. jordani* yielded about a 10% mating success rate, while the reverse cross yielded only a 1% success rate. (It should be noted that laboratory crosses between homospecifics yields only about a 5% mating success rate for *P. jordani* and a 16% success rate for *P. metcalfi*.) These data indicate strong mating asymmetry, and may have given rise to the pattern of differential introgression observed in the Balsam Mountain transect.

A second possible mechanism for hybrid zone movement is dispersal asymmetry. Asymmetrical dispersal of female *P. metcalfi* would result in a pattern of displacement of *P. metcalfi* mtDNA relative to that of *P. metcalfi* nuclear DNA and morphology. While no data are available on dispersal of *P. jordani*, *P. metcalfi*, and *P. teyahalee*, numerous studies have estimated the related measure of home range size (Table 6). Madison and Shoop (1970) found that *P. metcalfi* males had home ranges that were five times greater than those estimated for females, although sample sizes used in this study were small ($n_{males} = 2$, $n_{females} = 4$). Studies on *P. jordani* and *P. teyahalee* showed asymmetries between the sexes that are similar in magnitude and direction (Merchant 1972, Nishikawa 1990). Given the strength and direction of asymmetry in home range size, it is unlikely that asymmetrical dispersal of female *P. metcalfi* is a valid explanation for the pattern of hybrid zone movement observed in this study.

Similar hypotheses of hybrid zone movement have been proposed by other researchers. García-París et al. (2003) documented a moving hybrid zone between populations of fire salamanders of the genus *Salamandra* on the Iberian Peninsula. In that study, the authors found strong discordance between mtDNA on the one hand and allozymes, morphology, and life history on the other. Similarly to the study presented here, male-biased dispersal in *Salamandra* rules out the possibility that female dispersal played a role in the observed pattern of differential introgression. The authors also conclude that sexual asymmetry is likely important, but provide no definitive evidence for it. In another study, Rohwer et al. (2001) examined hybridization between warbler species of the genus *Dendroica* from the Pacific Northwest. Patterns of differential introgression between mtDNA and a suite of morphological markers led the researchers to conclude that the hybrid zone is moving. Independent observations on behavior and inferences from historical data suggest mating asymmetry may be the cause of hybrid zone movement in that system.

Hairston et al. (1992) made the assumption that red cheek pigmentation is neutrally diffusing across the *P. jordani-P. metcalfi* hybrid zone. Based on the most recent warming period during the Hypsithermal Interval (~5,000 years ago; see Pielou 1991), the authors hypothesized that populations of *P. jordani* and *P. metcalfi* migrated along the Balsam Mountain ridgeline from their mountain top refuges and met near the center of the hybrid zone as inferred by the authors. However, the expanded and finer-scale sampling performed in this study uncovered extensive hybridization farther north than that documented by Hairston et al. (1992), thus nearly doubling the width of the hybrid zone. Furthermore, this study documents a non-clinal transition between the parental species, which suggests a much more complex biogeographic history than the one outlined by Hairston et al. (1992). A more likely scenario is that repeated bouts of isolation and secondary contact throughout the Pleistocene (perhaps as early as eight million years ago, i.e., shortly after molecular clock estimates place the date of divergence; Highton 1995, Kozak et al. 2005, Wiens et al. 2006), have left a more complex, geographically reticulate pattern of hybridization.

Patterns of differential introgression seen in the Palmer Creek and Mt Sterling transects suggest *P. jordani* may be expanding downslope into the range of *P. teyahalee* as well. The dynamics of hybridization, however, may differ somewhat from those found between *P. jordani* and *P. metcalfi*. First, there is likely to be differential adaptation of the parental species because *P. teyahalee* is restricted to low elevations, while *P. jordani* and *P. metcalfi* are only found at high elevations; the parental species belong to different species complexes (*P. jordani* and *P. metcalfi* belong to the *P. jordani* complex and *P. teyahalee* belongs to the *P. glutinosus* complex) and are morphologically more differentiated from one another than *P. jordani* is from *P. metcalfi* (Highton and Peabody 2000); and previous studies (Hairston 1980a, 1980b, 1983a, Hairston et al. 1987, Adams 2004) have demonstrated that competitive interactions play a role in the distributions of *P. jordani* and *P. teyahalee*. Second, *P. jordani* and *P. metcalfi* occur at a greater density than *P. teyahalee* (Highton 1970, Merchant 1972, Hairston 1980a, 1980b). Population density is one determinant of hybrid zone structure and, when asymmetrical, may result in the movement of the hybrid zone towards the less dense parental species (Barton and Hewitt 1985).

An additional line of evidence that the hybrid zone between *P. jordani* and *P. metcalfi* is dynamic comes from ecological niche modeling. A fine-scale analysis of habitat suitability values along the Balsam Mountain transect (Figures 3.3 and 3.7) suggests the hybrid zone may lie in an ecotonal area where highly suitable habitats between *P. jordani* and *P. metcalfi* meet. Models depict highly suitable *P. metcalfi* habitat at areas surrounding, but not including, the highest elevations of the Balsam Mountain ridgeline. The highest elevations are currently occupied by populations containing predominantly *P. jordani* mtDNA, nuclear DNA, and morphology. Recent work suggests the ranges of *P. jordani* and *P. metcalfi* are influenced by climate (Chapter

IV). Therefore, the observed mtDNA “footprint” may reflect a past distribution of *P. metcalfi*, with movement of the hybrid zone tracking changes in suitable habitat.

The results of the ecological niche modeling highlight the importance of elevation in determining the position and structure of the hybrid zone. Climatic conditions along elevational gradients are likely to involve temperature and moisture, two variables that have been implicated in amphibian range restrictions in previous studies (Daszak et al. 2005, McMenamin et al. 2008). In this study, the two variables with the greatest explanatory power, precipitation in the driest month (BIO14) and precipitation in the driest quarter (BIO17), clearly indicate that moisture is important in determining the ranges of *P. jordani* and *P. metcalfi*. Further support for the importance of elevation in determining the structure of the hybrid zone comes from the presence of *P. teyahalee* in the vicinity of Pin Oak Gap. At an elevation of 1,348 m, Pin Oak Gap is a low point in the otherwise high elevation of the Balsam Mountain ridgeline, and the presence of *P. teyahalee* in this area suggests *P. jordani* and *P. metcalfi* may be at a competitive disadvantage.

One interesting observation is that early studies on *P. jordani* morphology show that red cheek pigmentation has an aposematic function, serving as a warning to potential predators (Huheey 1960, Brodie and Howard 1973, Hensel and Brodie 1976). While not toxic, all three species are noxious, and when disturbed, such as during a predator attack, copious amounts of glandular secretions are released from the skin (Huheey 1960, Brodie and Howard 1973, Hensel and Brodie 1976, Brodie et al. 1979, pers. obs.). Another plethodontid salamander, *Desmognathus imitator*, is likely a Batesian mimic of *P. jordani*, with about 25% of the population possessing red cheeks like their noxious model

(Orr 1968, Brodie and Howard 1973). Aposematism and mimicry of red cheek pigmentation suggests this trait is under positive selection, and hybrids possessing at least some red pigmentation may have a selective advantage. If this were the case, increased fitness of red-cheeked populations may be another explanation for the patterns of introgression observed in this study. Additional work on the introgression of this morphological trait is needed.

The results presented here suggest that the range of *P. jordani* is currently expanding southwards, and possibly downslope as well. This finding reflects a complex and dynamic biogeographic history of repeated isolation and contact that likely results from climatic oscillations and shifting habitat. Accordingly, the extensive occurrence of *P. metcalfi* mtDNA may best be viewed as a relict, i.e, a footprint of the historic range of *P. metcalfi* that cannot be eliminated because of mating asymmetry. Other studies have documented the same phenomenon of a moving hybrid zone that leaves a “wake” of mtDNA occupying the former range of the retreating species (Rohwer et al. 2001, García-París et al. 2003, Leaché and Cole 2007). Furthermore, severely limited dispersal abilities (as suggested by small home range sizes; Table 6), long generation times (every other year beginning at four years of age for *P. jordani* and *P. metcalfi*; Hairston 1983b), and stasis during the 18-year study period of Hairston et al. (1992) suggest this hybrid zone may be moving very slowly. Results from the ecological niche modeling further suggest that the hybrid zone may shift in response to changing climates, continually tracking suitable habitats. Hairston et al.’s (1992) hypothesis of neutral diffusion gives way to one of hybrid zone movement and differential introgression on an evolutionary time scale not readily measured by ecological studies (Dasmahapatra et al. 2002).

Figure 3.1. (A) Map of study area showing collection localities of parental (*Plethodon jordani*, *P. metcalfi*, and *P. teyahalee*) and hybrid samples. Inset depicts location of study within the continental United States. (B) Expanded map of hybrid zones from A showing collection localities of hybrid samples. In both A and B, darkened areas = high elevation and light areas = low elevation.

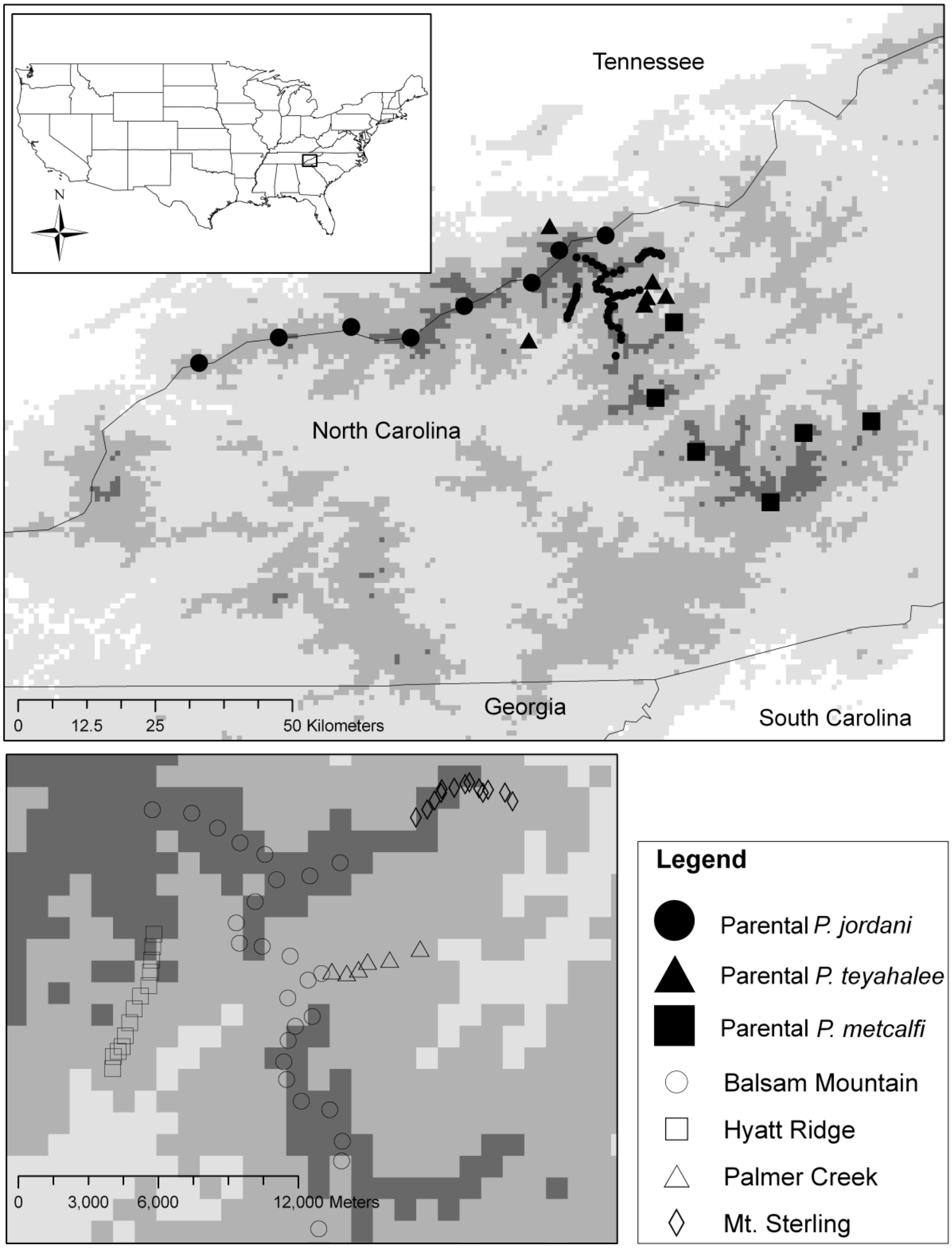

Tennessee
N
North Carolina
Georgia
South Carolina
0
12.5
25
50 Kilometers
0
3,000
6,000
12,000 Meters
Legend
● Parental *P. jordani*
▲ Parental *P. teyahalee*
■ Parental *P. metcalfi*
○ Balsam Mountain
□ Hyatt Ridge
△ Palmer Creek
◊ Mt. Sterling

Figure 3.2. (A) Map of the mtDNA ND2 gene with adjacent tRNA genes and relative positions of forward (MC001) and reverse (MC002) primers. Expanded sections of the gene show the six base pair restriction enzyme (*BanI* and *MfeI*) recognition sites and cut sites (indicated by "/"). (B) Partial map of nuclear ILF3 gene showing relative positions of the middle exon and two introns and the forward (MC003) and reverse (MC004) primers. Expanded section depicts SNPs (in bold) that, when used in tandem, are diagnostic for *Plethodon jordani*, *P. metcalfi*, and *P. teyahalee*. Base pairs are indicated below arrows in both A and B. Maps are not drawn to scale.

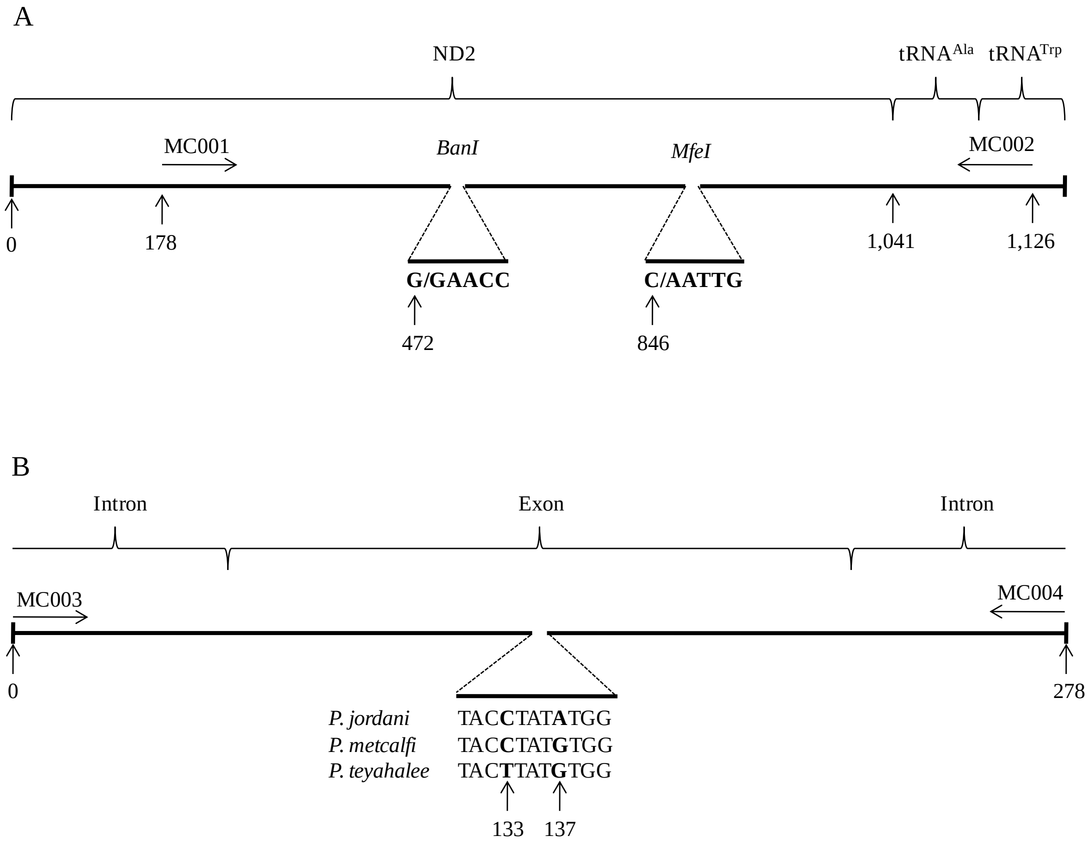
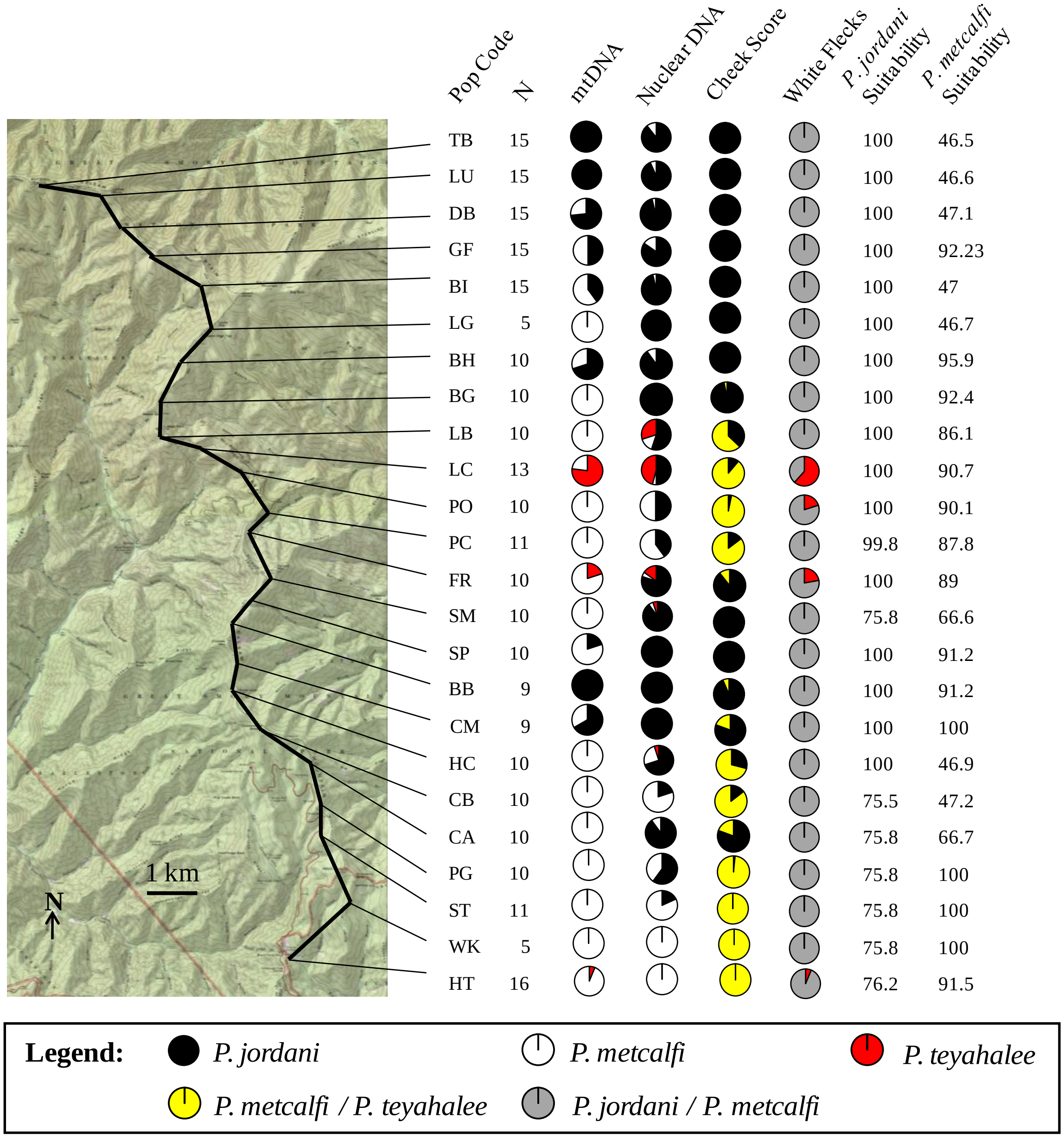

A
ND2
tRNA^{Ala}
tRNA^{Trp}
MC001
BanI
MfeI
MC002
0
178
1,041
1,126
G/GAACC
C/AATTG
472
846
B
Intron
Exon
Intron
MC003
MC004
0
278
P. jordani TACCTATATGG
P. metcalfi TACCTATGTGG
P. teyahalee TACTTATGTGG
133
137

Figure 3.3. Map of Balsam Mountain showing transect (bold line), collection localities, sample sizes, marker scores, and habitat suitability values. Pie charts are interpreted as follows: (1) proportion of samples at a given locality that are diagnostic for each parental species, (2) proportion of alleles at a given locality that are diagnostic for each parental species, (3) the average scaled cheek pigmentation score (i.e., average score divided by the average for pure parental *Plethodon jordani*), and (4) the proportion of animals that have at least some white flecks. Habitat suitability values are extracted from the ecological niche models presented in Figure 3.7 and are given as a percentage from 0 to 100. Note that more than one collection locality may lie within a single grid cell; therefore, identical values may not be independent from one another.



Pop Code	N	*P. jordani* Suitability	*P. metcalfi* Suitability
TB	15	100	46.5
LU	15	100	46.6
DB	15	100	47.1
GF	15	100	92.23
BI	15	100	47
LG	5	100	46.7
BH	10	100	95.9
BG	10	100	92.4
LB	10	100	86.1
LC	13	100	90.7
PO	10	100	90.1
PC	11	99.8	87.8
FR	10	100	89
SM	10	75.8	66.6
SP	10	100	91.2
BB	9	100	91.2
CM	9	100	100
HC	10	100	46.9
CB	10	75.5	47.2
CA	10	75.8	66.7
PG	10	75.8	100
ST	11	75.8	100
WK	5	75.8	100
HT	16	76.2	91.5

Figure 3.4. Map of Hyatt Ridge showing transect (bold line), collection localities, sample sizes, and marker scores. Interpretation of marker scores is as given in Figure 3.3.

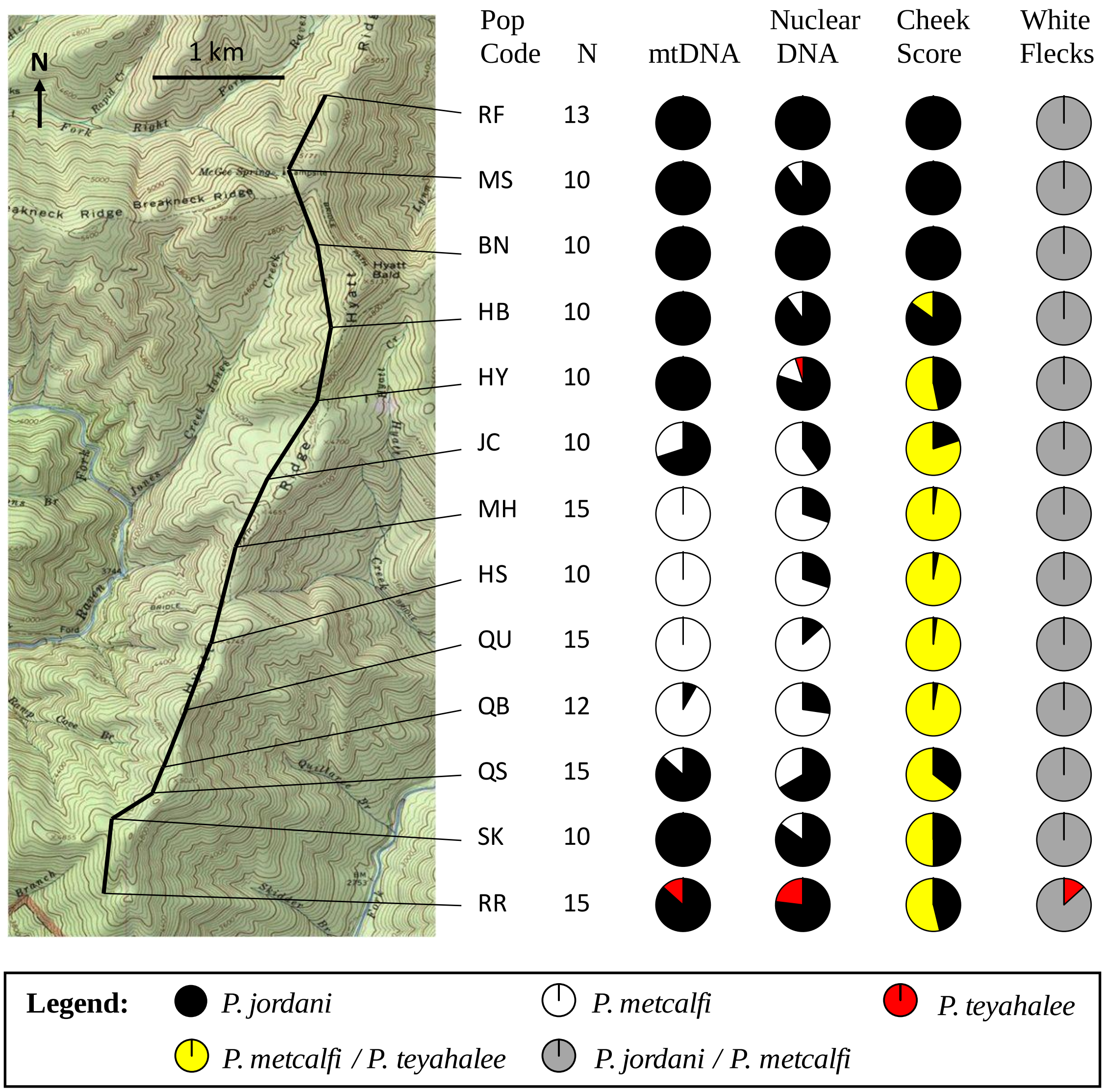

1 km
N
Pop Code
N
mtDNA
Nuclear DNA
Cheek Score
White Flecks
RF 13
MS 10
BN 10
HB 10
HY 10
JC 10
MH 15
HS 10
QU 15
QB 12
QS 15
SK 10
RR 15
Legend:
P. jordani
P. metcalfi
P. teyahalee
P. metcalfi / P. teyahalee
P. jordani / P. metcalfi

Figure 3.5. Map of Palmer Creek showing transect (bold line), collection localities, sample sizes, and marker scores. Interpretation of marker scores is as given in Figure 3.3.

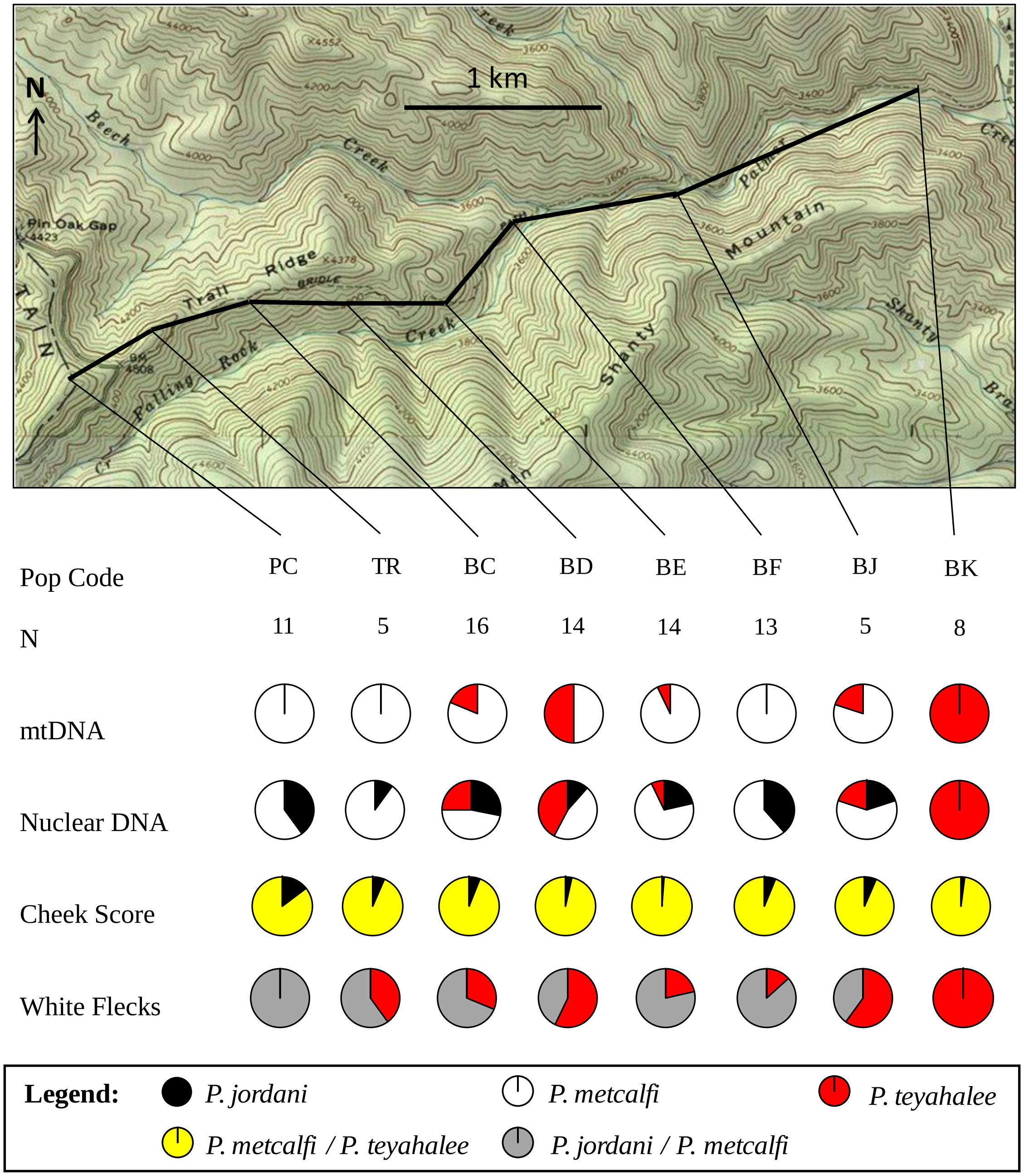

N
1 km
Beech
Creek
Pin Oak Gap
Trail
Ridge
Falling Rock
Creek
Palmer
Mountain
Shanty
Shanty
Pop Code
PC
TR
BC
BD
BE
BF
BJ
BK
N
11
5
16
14
14
13
5
8
mtDNA
Nuclear DNA
Cheek Score
White Flecks
Legend:
P. jordani
P. metcalfi
P. teyahalee
P. metcalfi / P. teyahalee
P. jordani / P. metcalfi

Figure 3.6. Map of Mt Sterling showing transect (bold line), collection localities, sample sizes, and marker scores. Interpretation of marker scores is as given in Figure 3.3.

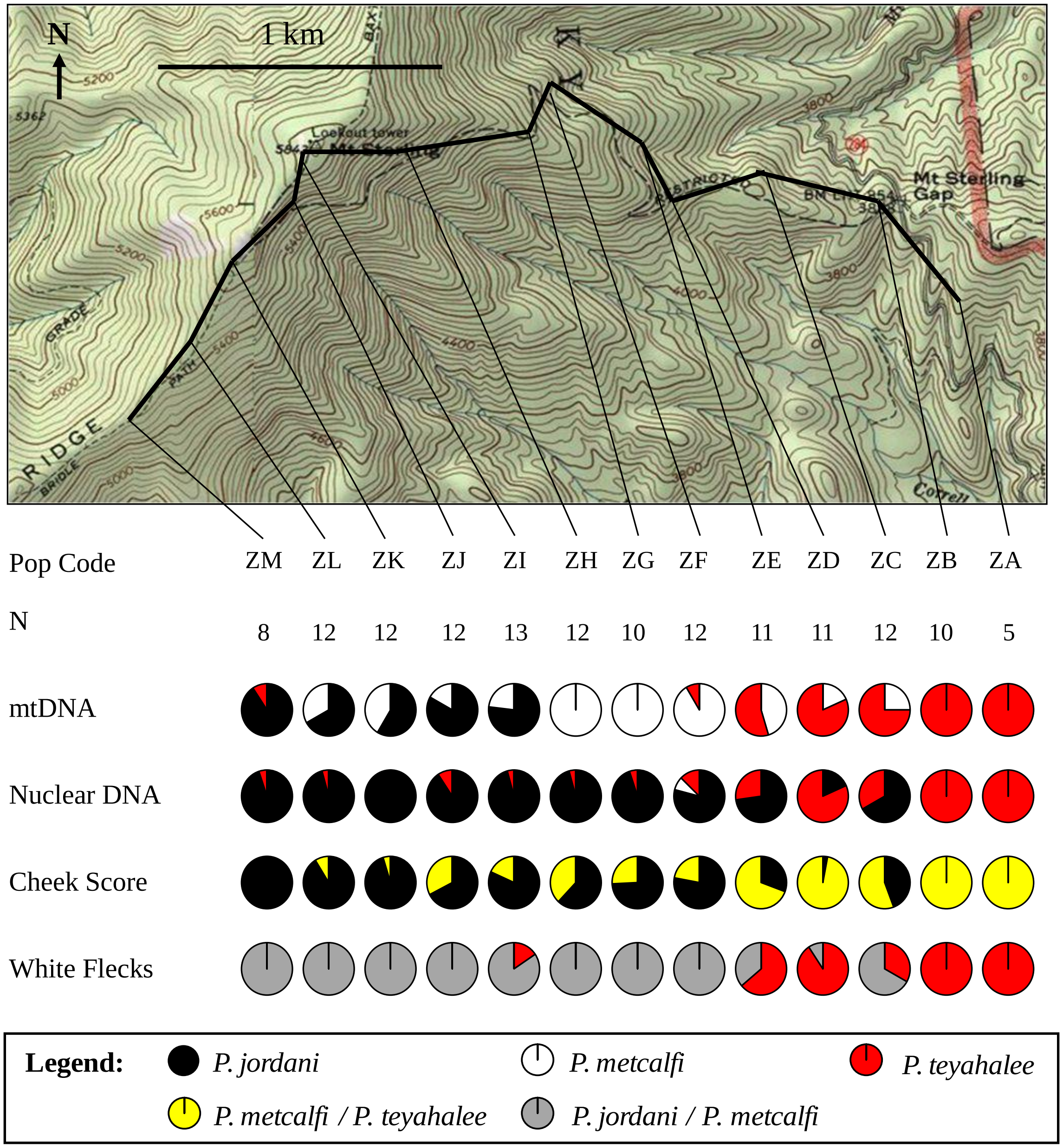
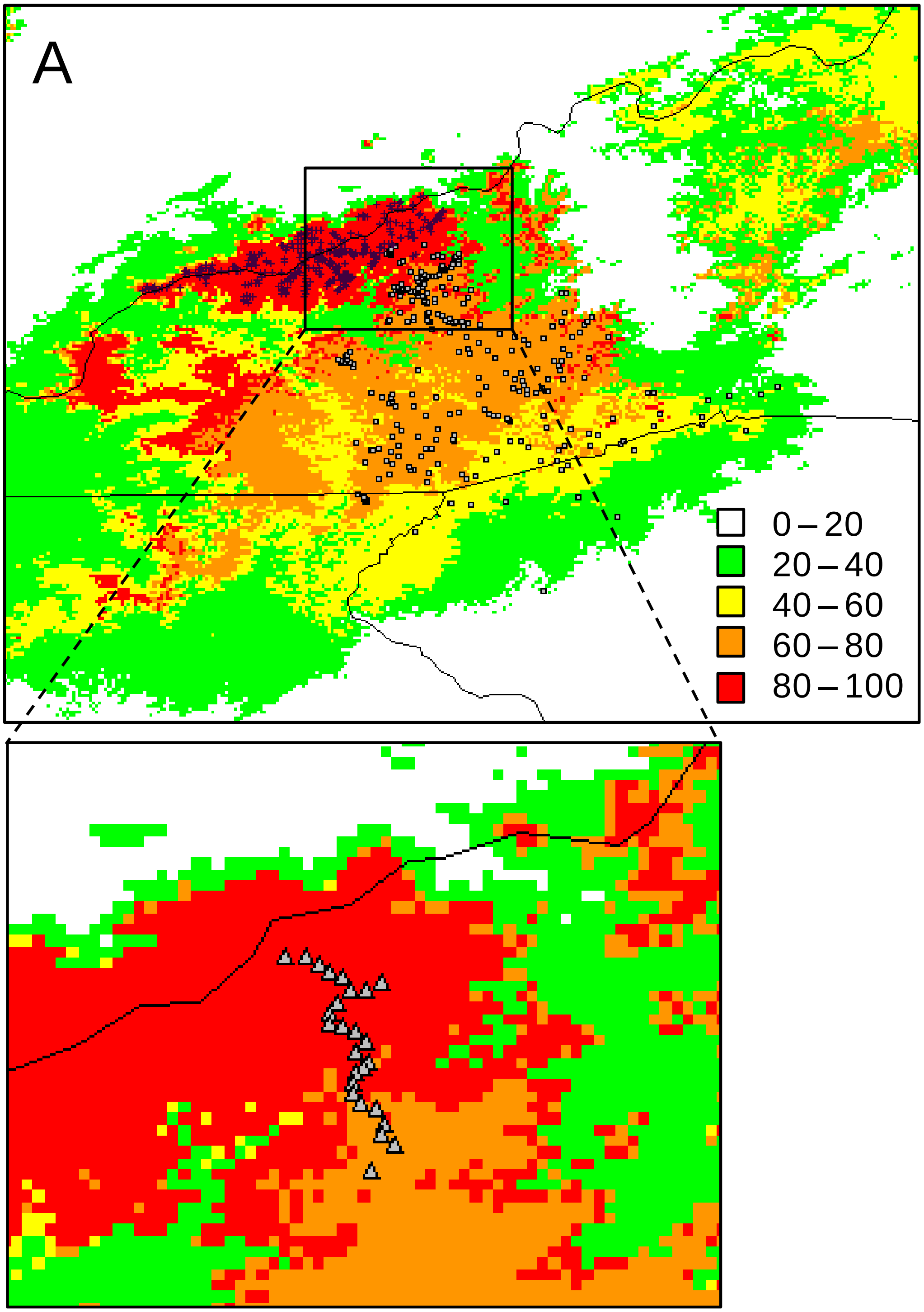

N
1 km
Lookout tower
Mt Sterling
Mt Sterling Gap
RIDGE
BRIDLE
PATH
GRADE
Correll
Pop Code
ZM ZL ZK ZJ ZI ZH ZG ZF ZE ZD ZC ZB ZA
N
8 12 12 12 13 12 10 12 11 11 12 10 5
mtDNA
Nuclear DNA
Cheek Score
White Flecks
Legend:
P. jordani
P. metcalfi
P. teyahalee
P. metcalfi / P. teyahalee
P. jordani / P. metcalfi

Figure 3.7. Ecological niche modeling results showing present-day predicted geographic distributions for *P. jordani* (A) and *P. metcalfi* (B). For comparison, collection localities are shown for both *P. jordani* (+) and *P. metcalfi* (□). Inner boxes encompass hybrid zone and are expanded to show sampling localities (Δ) along the Balsam Mountain transect. Colors indicate habitat suitability values as assigned by Maxent, and are given as percentages (cumulative probability x 100; see text for more detail).


A
0 – 20
20 – 40
40 – 60
60 – 80
80 – 100

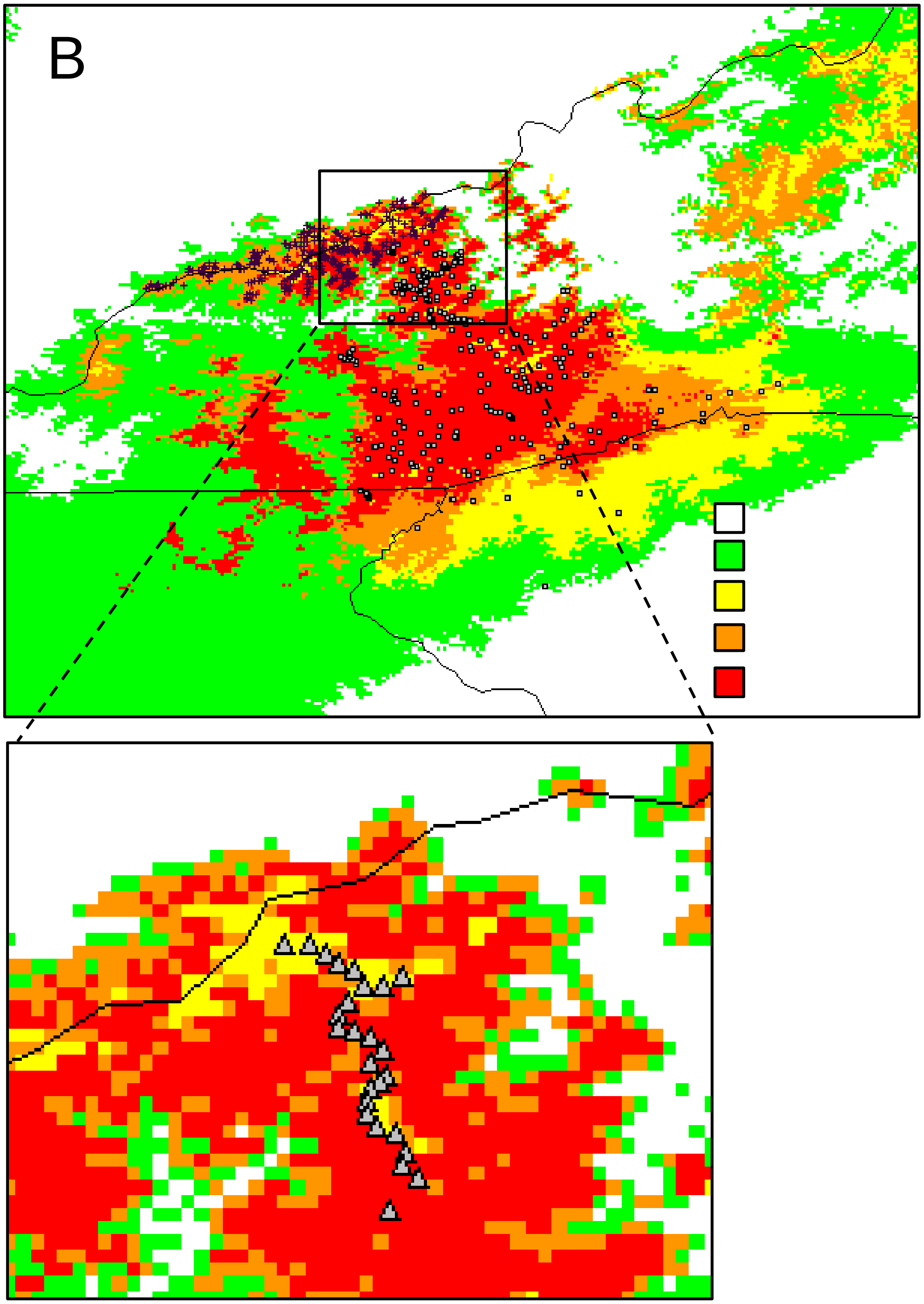

B

Table 3.1. Climatic variables[1] used in creation of ecological niche models.

Variables[2]	Description	Percent for *P. jordani*[3]	Percent for *P. metcalfi*[3]
BIO1	Annual Mean Temperature	3.3	3.5
BIO2	Mean Diurnal Range	0.2	0
BIO3	Isothermality	0	0.1
BIO4	Temperature Seasonality	1.1	1
BIO5	Max Temperature of Warmest Month	0	0
BIO6	Min Temperature of Coldest Month	0	0
BIO7	Temperature Annual Range	0.1	0
BIO8	Mean Temperature of Wettest Quarter	2.1	0.2
BIO9	Mean Temperature of Driest Quarter	0	0.5
BIO10	Mean Temperature of Warmest Quarter	2	1.5
BIO11	Mean Temperature of Coldest Quarter	0.2	0
BIO12	Annual Precipitation	0.6	0.2
BIO13	Precipitation of Wettest Month	0.2	0.1
BIO14	Precipitation of Driest Month	19.4	53.4
BIO15	Precipitation Seasonality	6.9	7.9
BIO16	Precipitation of Wettest Quarter	0	0
BIO17	Precipitation of Driest Quarter	58	31.3
BIO18	Precipitation of Warmest Quarter	5.4	0
BIO19	Precipitation of Coldest Quarter	0.3	0.5

[1] Variables are as given on the WorldClim database, available at www.worldclim.org/bioclim.htm.

[2] Variable names are as assigned by WorldClim.

[3] Given as a percent contribution for model predictions for *Plethodon jordani* and *P. metcalfi*.

Table 3.2. Summary of salamander samples from the hybrid zone that are classified by their nuclear and mtDNA haplotypes. The number of animals with at least some red on their cheeks and with at least some white flecks are given in parentheses, respectively.

	mtDNA					
Nuclear DNA	*P. jordani*		*P. metcalfi*		*P. teyahalee*	
P. jordani / *P. jordani*	180	(177,1)	168	(142,2)	15	(10,6)
P. metcalfi / *P. metcalfi*	4	(3,0)	96	(12,3)	2	(0,2)
P. teyahalee / *P. teyahalee*	0	(0,0)	5	(3,1)	47	(2,42)
P. jordani / *P. metcalfi*	28	(27,0)	67	(25,3)	1	(0,0)
P. jordani / *P. teyahalee*	8	(8,1)	15	(10,2)	9	(5,5)
P. metcalfi / *P. teyahalee*	0	(0,0)	0	(0,0)	0	(0,0)

Table 3.3. Contingency table showing individual sample data used to test for differential patterns of introgression.

	mtDNA		nuclear DNA		
Species	Obs.	Exp.	Obs.	Exp.	Total
Plethodon jordani	227	365	868	730	1095
P. metcalfi	369	235	336	470	705
P. teyahalee	82	78	152	156	234
Total	678	678	1356	1356	2034

Table 3.4. Contingency table showing sample localities classified by their most common haplotype.

	mtDNA		nuclear DNA		
Species	Obs.	Exp.	Obs.	Exp.	Total
Plethodon jordani	27	36	45	36	72
P. metcalfi	35	27.5	20	27.5	55
P. teyahalee	12	10.5	9	10.5	21
Total	74	74	74	74	148

Table 3.5. Results of general linear models (GLMs) showing the Akaike information criterion values (given as ΔAIC), residual deviances (and associated degrees of freedom), and significance levels.

Model	ΔAIC[1]	Residual Deviance[2]	D.F.[3]	P-value[4]
Null	387.3449	405.4704	10	6.4467×10^{-81}
Symmetrical	55.6383	69.7638	8	5.4763×10^{-12}
Asymmetrical[5]				
P. metcalfi / *P. teyahalee*	-	-	-	-
P. metcalfi / *P. metcalfi*	57.0183	69.1439	7	2.2001×10^{-12}
P. jordani / *P. metcalfi*	54.0597	66.1852	7	8.6866×10^{-12}
P. jordani / *P. teyahalee*	54.0597	66.1852	7	8.6866×10^{-12}
P. jordani	15.8975	24.0230	5	2.1491×10^{-4}
P. metcalfi	13.2398	21.3654	5	6.9090×10^{-4}
P. jordani / *P. jordani*	12.8195	24.9450	7	7.7603×10^{-4}
P. teyahalee / *P. teyahalee*	1.8222	13.9477	7	**0.052120**
P. teyahalee	0.0000	8.1255	5	**0.14950**

[1] Akaike information criterion values given as the difference from the best model.

[2] Residual deviance is a measure of the goodness-of-fit of a model to the data. Higher values indicate more variation is unaccounted for by the model and, therefore, lower values indicate models with a better fit.

[3] Degrees of freedom of the residual deviance.

[4] Insignificant residual deviance (P > 0.05) means the residual variance is adequately explained as sampling error and, therefore, the model is an adequate description of the data. Insignificant residual P-values are given in bold.

[5] Results for all nine possible asymmetrical models are given (except for *P. metcalfi* / *P. teyahalee* heterozygotes since none were found at the nuclear marker).

Table 3.6. Home range sizes in m^2 (sample sizes given in parentheses) for males and females of *Plethodon jordani*, *P. teyahalee*, and *P. metcalfi*.

Species	Males	Females	Reference
P. jordani	5.0 (3)	1.9 (4)	Nishikawa 1990
	11.5 (>10)	2.8 (>10)	Merchant 1972
P. teyahalee	0.5 (3)	1.0 (8)	Nishikawa 1990
	14.4 (<10)	6.5 (>10)	Merchant 1972
P. metcalfi	51.5 (2)	11.8 (4)	Madison and Shoop 1970

Appendix 3.1. Collection localities, sites codes, elevation, and latitude and longitude for animals captured in the hybrid zones.

Transect	Site Code	Elev. (m)	Latitude	Longitude
Balsam Mountain	PG	1609	35.56391995	-83.16177777
	CA	1719	35.57600245	-83.16661544
	SM	1719	35.61182943	-83.17341010
	PC	1390	35.62587308	-83.17494958
	PO	1402	35.63506813	-83.18189032
	BG	1597	35.64780588	-83.20261272
	BB	1743	35.60265632	-83.18266536
	CM	1780	35.59448665	-83.18429759
	HC	1707	35.58762766	-83.18325042
	CB	1792	35.57927508	-83.17757258
	LC	1475	35.63881075	-83.19269241
	FR	1402	35.61897075	-83.18275550
	LB	1548	35.64003041	-83.20141642
	SP	1707	35.60772728	-83.17742746
	BH	1707	35.65601586	-83.19533869
	LG	1670	35.66442415	-83.18705236
	ST	1597	35.55633270	-83.16211448
	DB	1768	35.68424458	-83.20981273
	BI	1707	35.67418439	-83.19162726
	HT	1402	35.53020408	-83.17087478
	LU	1829	35.68998283	-83.21979420
	TB	1804	35.69133307	-83.23495880
	GF	1634	35.67858068	-83.20113841
	WK	1634	35.54805040	-83.15396128
Hyatt Ridge	HS	1414	35.60886296	-83.24311502
	JC	1719	35.61778960	-83.23911412
	HB	1500	35.62823755	-83.23473519
	MS	1536	35.63775908	-83.23799285
	QB	1512	35.60026432	-83.24643994
	SK	1500	35.59652094	-83.24994428
	RR	1378	35.59175893	-83.25007582
	MH	1390	35.61473777	-83.24179986
	QS	1512	35.59844068	-83.24803346
	QU	1524	35.60440438	-83.24534156
	HY	1414	35.62363991	-83.23620805
	RF	1481	35.64342471	-83.23435206
	BN	1536	35.63347478	-83.23522333
Palmer Creek	BE	1122	35.63002728	-83.15551872
	PC	1390	35.62587308	-83.17494958
	TR	1305	35.62835489	-83.16980536
	BD	1219	35.62859372	-83.16005132
	BC	1280	35.62936480	-83.16579152
	BF	1061	35.63301985	-83.15201048

	BJ	1000	35.63377620	-83.14348422
	BK	951	35.63799563	-83.13179235
Mt Sterling	ZI	1768	35.69926099	-83.12346552
	ZB	1207	35.69795847	-83.09910776
	ZE	1451	35.69957733	-83.10909630
	ZD	1329	35.69787315	-83.10758163
	ZL	1585	35.69144561	-83.12905953
	ZM	1561	35.68843010	-83.13338816
	ZK	1646	35.69491291	-83.12619018
	ZJ	1695	35.69779646	-83.12370294
	ZH	1622	35.69982399	-83.11864002
	ZF	1451	35.70187413	-83.11279277
	ZG	1536	35.70117879	-83.11439897
	ZC	1280	35.69900670	-83.10565542
	ZA	1134	35.69457493	-83.09619011

Appendix 3.2. Collection localities, sites codes, elevation, and latitude and longitude for parental taxa.

Species	Site Code	Elev. (m)	Latitude	Longitude
Plethodon jordani	SH	1600	35.61192974	-83.41908953
	JR	1524	35.56044427	-83.72307361
	PE	1600	35.65003605	-83.30803398
	GU	1951	35.70336446	-83.26300058
	CS	1539	35.57753645	-83.60428684
	VF	1353	35.51853193	-83.85393919
	MB	1978	35.56004153	-83.50680322
	CO	1573	35.72775821	-83.18706962
P. metcalfi	WK	1609	35.54654147	-83.15465992
	WR	1707	35.46220459	-83.13776387
	TA	1695	35.29046957	-82.91683637
	MP	1561	35.42294145	-82.75138204
	CL	1631	35.40413851	-82.86246472
	LK	1707	35.37606578	-83.03479743
P. teyahalee	CK	1707	35.60772728	-83.17742746
	VA	863	35.74326344	-83.27905659
	VB	671	35.55596160	-83.31333018
	VD	1329	35.62904920	-83.08786358
	VE	881	35.61496116	-83.12378423
	SB	975	35.62684900	-83.11931790

Appendix 3.3. Pure parental individuals (*Plethodon jordani*, *P. metcalfi*, and *P. teyahalee*) and marker scores used in panel for marker development.

Species	Site Code[1]	Indiv. Code[2]	SVL (mm)[3]	Cheek Score[4]	White Flecks	mtDNA[5]	nDNA[6]
P. jordani	MB	97	55	10	Absent	J	n/a
		98	39	4	Absent	J	n/a
	CO	306	43	3	Absent	J	n/a
		307	48	10	Absent	J	n/a
	SH	332	58	11	Absent	J	J/J
		333	48	7	Absent	J	J/J
	JR	340	41	7	Absent	J	J/J
		341	42	11	Absent	J	J/J
	PE	402	43	7	Absent	J	J/J
		403	41	4	Absent	J	J/J
	GU	435	47	12	Absent	J	n/a
		436	55	12	Absent	J	J/J
		437	36	6	Absent	J	n/a
		438	67	8	Absent	J	J/J
	CS	447	43	4	Absent	J	J/J
		448	42	9	Absent	J	J/J
	VF	942	43	3	Absent	J	J/J
		944	47	4	Absent	J	J/J
P. metcalfi	WK	64	53	0	Absent	M	M/M
		65	60	0	Absent	M	M/M
		66	65	0	Absent	M	M/M
	WR	201	52	0	Absent	M	n/a
		202	31	0	Absent	M	n/a
		203	29	0	Absent	M	M/M
	TA	372	49	0	Absent	M	n/a
		373	38	0	Absent	M	M/M
		374	32	0	Absent	M	M/M
		375	45	0	Absent	M	n/a
		376	48	0	Absent	M	M/M
		377	54	0	Absent	M	n/a
	MP	382	52	0	Absent	M	M/M
		383	31	0	Absent	M	M/M
	CL	424	30	0	Absent	M	M/M
		425	50	0	Absent	M	M/M
		426	55	0	Absent	M	n/a
		427	31	0	Absent	M	n/a
	LK	365	54	0	Absent	M	n/a
		366	50	0	Absent	M	n/a
		367	49	0	Absent	M	n/a
P. teyahalee	CK	222	66	0	Present	T	T/T
		223	27	0	Present	T	n/a
		224	44	0	Present	T	T/T
	VA	902	55	0	Present	T	T/T
	VB	911	66	0	Present	T	T/T
		912	65	0	Present	T	T/T
		914	60	0	Present	T	n/a
	VD	932	44	0	Present	T	T/T
	VE	935	58	0	Present	T	T/T
		937	43	0	Present	T	T/T
		938	42	0	Present	T	n/a

SB	327	19	0	Present	T	n/a
	329	69	0	Present	T	n/a

[1] See Figure 1 and Appendix 1 for site locations.
[2] Individual codes are those assigned by the author.
[3] SVL (Snout Vent Length) was measured from the tip of the snout to the posterior end of the cloaca.
[4] Cheek pigmentation was scored on a scale of 0 to 13, with 0 indicating a complete lack of red pigmentation and 13 indicating extensive and bright red pigment.
[5] As the mitochondrial genome is haploid, only a single haplotype is given.
[6] As the nuclear genome is diploid, two haplotypes are given.

Appendix 3.4. Samples (arranged by transect) and marker scores for all samples used in analyses.

Transect	Site Code[1]	Indiv. Code[2]	SVL (mm)[3]	Cheek Score[4]	White Flecks	mtDNA[5]	nDNA[6]
Balsam Mtn	PG	1	35	0	Absent	M	J/M
		2	50	0	Absent	M	M/M
		3	52	0	Absent	M	J/J
		4	36	0	Absent	M	J/J
		5	58	0	Absent	M	J/M
		6	54	0	Absent	M	J/J
		7	48	0	Absent	M	J/M
		8	50	0	Absent	M	J/M
		9	54	1	Absent	M	J/J
		10	57	0	Absent	M	M/M
	CA	11	38	0	Absent	M	J/J
		12	37	3	Absent	M	J/J
		13	35	6	Absent	M	J/J
		14	40	0	Absent	M	J/J
		15	36	7	Absent	M	J/J
		47	50	11	Absent	M	J/J
		48	37	6	Absent	M	J/M
		49	45	3	Absent	M	J/J
		50	52	9	Absent	M	J/M
		51	60	5	Absent	M	J/J
	SM	16	54	9	Absent	M	J/M
		17	58	0	Absent	M	J/J
		18	53	10	Absent	M	J/J
		19	41	2	Absent	M	J/J
		20	57	11	Absent	M	J/J
		21	43	9	Absent	M	J/J
		22	50	11	Absent	M	J/J
		23	49	10	Absent	M	J/T
		24	51	9	Present	M	J/J
		25	32	1	Absent	M	J/J
	PC	26	30	0	Absent	M	M/M
		27	38	1	Absent	M	J/J
		28	43	0	Absent	M	M/M
		29	52	0	Absent	M	J/M
		30	29	0	Absent	M	J/M
		32	45	0	Absent	M	J/M
		33	50	0	Absent	M	M/M
		34	47	0	Absent	M	J/M
		35	50	0	Absent	M	M/M
		36	50	9	Absent	M	J/J
	PO	37	53	0	Absent	M	J/J
		42	58	0	Absent	M	M/M
		43	44	0	Absent	M	J/M
		44	57	1	Absent	M	J/M
		45	32	0	Absent	M	J/M
		46	28	0	Absent	M	J/M
	BG	52	67	10	Absent	M	J/J
		53	63	7	Absent	M	J/J
		54	61	8	Absent	M	J/J
		55	38	12	Absent	M	J/J
		56	47	0	Absent	M	J/J

	57	60	5	Absent	M	J/J
	58	58	4	Absent	M	J/J
	59	64	2	Absent	M	J/T
	60	55	4	Absent	M	J/J
	61	33	8	Absent	M	J/J
BB	67	43	4	Absent	J	J/J
	69	27	7	Absent	J	J/J
	70	31	4	Absent	J	J/J
	71	31	1	Absent	J	J/J
	72	48	5	Absent	J	J/J
	73	59	11	Absent	J	J/J
	74	29	11	Absent	J	J/J
	75	54	5	Absent	J	J/J
CM	77	60	3	Absent	J	J/J
	78	54	1	Absent	J	J/J
	79	43	1	Absent	M	J/J
	80	55	10	Absent	M	J/J
	81	45	4	Absent	J	J/J
	82	70	11	Absent	M	J/J
	83	58	6	Absent	J	J/J
	84	46	2	Absent	J	J/J
	85	60	3	Absent	J	J/J
HC	132	33	0	Absent	M	J/M
	133	48	0	Absent	M	J/T
	134	33	6	Absent	M	J/J
	135	58	1	Absent	M	J/M
	136	41	3	Absent	M	J/M
	137	47	1	Absent	M	J/J
	138	47	7	Absent	M	J/M
	139	63	0	Absent	M	J/M
	140	66	0	Absent	M	J/J
	141	45	0	Absent	M	J/J
CB	142	54	0	Absent	M	M/M
	143	48	4	Absent	M	J/J
	144	60	0	Absent	M	M/M
	145	48	0	Absent	M	M/M
	146	65	0	Absent	M	M/M
	147	61	0	Absent	M	M/M
	148	55	0	Absent	M	M/M
	149	45	5	Absent	M	J/J
	150	47	0	Absent	M	M/M
	151	57	0	Absent	M	M/M
LC	152	58	0	Present	T	J/J
	153	56	2	Present	T	J/J
	154	42	2	Absent	M	J/J
	155	47	1	Absent	M	J/J
	156	62	0	Present	T	T/T
	157	37	0	Present	T	T/T
	159	38	0	Present	T	J/J
	160	41	1	Present	M	T/T
	161	40	1	Present	T	T/T
	193	41	0	Absent	T	T/T
	194	39	0	Absent	T	J/T
	195	55	0	Absent	T	J/M
FR	167	62	11	Absent	M	J/J
	168	60	4	Absent	M	J/T

	174	54	5	Absent	M	J/M
	175	60	12	Absent	M	J/J
	176	53	2	Absent	M	J/J
	177	50	0	Absent	M	J/T
	179	50	12	Absent	T	J/T
	180	57	12	Absent	M	J/J
	182	57	1	Absent	T	J/J
LB	183	57	10	Absent	M	J/M
	184	52	1	Absent	M	M/M
	185	56	1	Absent	M	J/M
	186	64	0	Present	M	J/M
	187	58	3	Absent	M	J/J
	188	33	0	Absent	M	J/J
	189	55	1	Absent	M	J/T
	190	55	0	Absent	M	J/J
	191	36	6	Absent	M	J/J
	192	36	1	Absent	M	J/J
SP	281	33	5	Present	M	J/J
	282	50	5	Absent	J	J/J
	283	50	9	Absent	J	J/J
	284	34	12	Absent	M	J/J
	285	32	2	Absent	M	J/J
	286	48	10	Absent	M	J/J
	287	45	12	Present	M	J/J
	288	49	13	Absent	M	J/J
	289	50	11	Absent	M	J/J
	290	56	8	Absent	M	J/J
BH	291	58	13	Absent	J	J/J
	292	57	8	Absent	J	J/J
	293	51	9	Absent	M	J/J
	294	52	6	Absent	M	J/J
	295	66	9	Absent	J	J/J
	296	62	6	Absent	J	J/J
	297	60	9	Absent	J	J/M
	298	63	3	Absent	J	J/J
	299	62	6	Absent	M	J/J
	300	51	8	Absent	J	J/M
LG	301	60	10	Absent	M	J/J
	302	60	12	Absent	M	J/J
	303	48	3	Absent	M	J/J
	304	50	4	Absent	M	J/J
	305	62	6	Absent	M	J/J
TR	317	41	0	Absent	M	M/M
	318	46	0	Absent	M	M/M
	319	50	1	Present	M	J/M
	320	42	0	Absent	M	M/M
	321	41	1	Present	M	M/M
ST	526	39	0	Absent	M	M/M
	527	32	0	Absent	M	M/M
	528	49	0	Absent	M	J/M
	529	53	0	Absent	M	M/M
	530	53	0	Absent	M	M/M
	531	42	0	Absent	M	M/M
	532	50	0	Absent	M	M/M
	533	38	0	Absent	M	M/M
	534	36	0	Absent	M	J/J

	535	44	0	Absent	M	J/M
	536	47	0	Absent	M	M/M
DB	537	53	7	Absent	J	J/J
	538	45	8	Absent	J	J/J
	540	48	4	Absent	M	J/J
	541	55	7	Absent	J	J/J
	542	54	11	Absent	J	J/M
	543	53	6	Absent	J	J/J
	544	44	4	Absent	J	J/J
	545	59	7	Absent	J	J/J
	546	62	10	Absent	J	J/J
	547	51	8	Absent	M	J/J
	548	49	9	Absent	J	J/J
	549	63	8	Absent	M	J/J
	550	54	11	Absent	J	J/J
	551	59	12	Absent	M	J/J
BI	552	36	9	Absent	M	J/J
	554	48	10	Absent	J	J/J
	555	32	10	Absent	J	J/J
	556	30	11	Absent	J	J/J
	557	34	9	Absent	J	J/J
	558	54	12	Absent	M	J/J
	559	54	10	Absent	M	J/J
	560	53	9	Absent	J	J/J
	561	60	11	Absent	M	J/J
	562	50	7	Absent	J	J/J
	563	53	10	Absent	J	J/J
	564	37	9	Absent	M	J/J
	565	30	9	Absent	M	J/M
	566	30	7	Absent	M	J/J
GF	567	43	12	Absent	J	J/M
	569	52	9	Absent	M	J/J
	570	55	10	Absent	M	J/J
	571	55	9	Absent	J	J/M
	572	54	12	Absent	J	J/M
	573	71	9	Absent	J	J/J
	574	53	10	Absent	M	J/M
	575	55	7	Absent	M	J/J
	576	47	9	Absent	M	J/J
	577	53	9	Absent	M	J/J
	579	63	8	Absent	J	J/J
	580	52	10	Absent	M	J/J
	581	46	12	Absent	J	J/J
HT	582	46	0	Absent	M	M/M
	583	41	0	Absent	M	M/M
	584	33	0	Absent	M	M/M
	585	53	0	Absent	M	M/M
	586	48	0	Absent	M	M/M
	587	49	0	Absent	M	M/M
	588	49	0	Absent	M	M/M
	589	34	0	Absent	M	M/M
	590	48	0	Absent	M	M/M
	591	51	0	Absent	M	M/M
	592	45	0	Absent	M	M/M
	593	58	0	Absent	M	M/M
	594	36	0	Absent	M	M/M

	595	40	0	Absent	M	M/M
	596	53	0	Absent	M	M/M
	597	65	0	Present	T	M/M
BU	665	54	6	Absent	M	J/J
	666	54	12	Absent	M	J/J
	667	56	7	Absent	M	J/M
	668	62	9	Absent	M	J/J
	669	63	8	Absent	M	J/J
	670	51	5	Absent	M	J/J
	671	56	11	Absent	M	J/J
	672	30	9	Absent	M	J/J
	673	59	10	Absent	M	J/J
	674	34	12	Absent	M	J/J
	675	50	10	Absent	M	J/J
	676	55	8	Absent	M	J/J
	677	45	10	Absent	M	J/J
	678	32	4	Absent	J	J/J
	679	59	12	Absent	J	J/J
BA	680	38	3	Absent	M	J/J
	681	62	13	Absent	M	J/J
	682	50	10	Absent	M	J/J
	683	53	10	Absent	M	J/J
	684	55	9	Absent	M	J/J
	685	37	5	Absent	M	J/J
	686	41	8	Absent	M	J/J
	687	56	10	Absent	M	J/J
	688	31	7	Absent	M	J/J
	689	54	10	Absent	M	J/J
	690	56	11	Absent	M	J/J
	691	53	6	Absent	M	J/J
	692	60	10	Absent	M	J/J
	694	37	6	Absent	M	J/J
LU	695	49	5	Absent	J	J/J
	696	55	7	Absent	J	J/J
	697	63	5	Absent	J	J/J
	698	53	6	Absent	J	J/J
	699	52	11	Absent	J	J/J
	700	54	10	Absent	J	J/J
	701	41	1	Absent	J	J/J
	702	64	9	Absent	J	J/J
	703	55	7	Absent	J	J/J
	704	50	6	Absent	J	J/J
	705	49	10	Absent	J	J/J
	706	54	9	Absent	J	J/J
	707	58	6	Absent	J	J/J
	708	53	6	Absent	J	J/M
	709	59	4	Absent	J	J/M
TB	710	40	6	Absent	J	J/M
	711	50	12	Absent	J	J/J
	712	44	4	Absent	J	J/J
	713	32	3	Absent	J	J/J
	714	44	6	Absent	J	J/J
	715	53	5	Absent	J	J/M
	716	39	9	Absent	J	J/J
	717	54	7	Absent	J	J/J
	719	55	6	Absent	J	J/J

		720	50	6	Absent	J	J/J
		721	47	7	Absent	J	J/J
		722	36	7	Absent	J	J/J
		723	57	11	Absent	J	J/J
		724	50	11	Absent	J	J/M
Hyatt Ridge	HS	231	52	2	Absent	M	J/M
		232	52	0	Absent	M	J/J
		233	45	0	Absent	M	J/M
		234	56	0	Absent	M	J/J
		235	47	0	Absent	M	M/M
		236	43	0	Absent	M	M/M
		237	38	0	Absent	M	M/M
		238	37	0	Absent	M	M/M
		239	42	0	Absent	M	M/M
		240	48	0	Absent	M	M/M
	JC	241	55	6	Absent	J	J/M
		242	35	1	Absent	J	J/M
		243	53	4	Absent	J	J/M
		244	31	1	Absent	J	J/M
		245	55	8	Absent	J	J/M
		246	55	0	Absent	J	J/M
		247	37	0	Absent	J	M/M
		248	32	0	Absent	M	J/M
		249	33	0	Absent	M	M/M
		250	28	0	Present	M	J/M
	HB	251	59	4	Absent	J	J/J
		252	59	1	Absent	J	J/J
		253	32	4	Present	J	J/J
		254	60	12	Present	J	J/M
		255	59	10	Absent	J	J/J
		256	65	4	Absent	J	J/J
		257	54	4	Absent	J	J/J
		258	55	6	Absent	J	J/J
		259	62	5	Absent	J	J/J
		260	48	4	Absent	J	J/M
	MS	261	49	10	Absent	J	J/J
		262	21	5	Absent	J	J/J
		263	51	6	Absent	J	J/J
		264	39	5	Absent	J	J/J
		265	53	10	Absent	J	J/J
		266	60	12	Present	J	J/J
		267	58	8	Absent	J	J/J
		268	52	8	Absent	J	J/J
		269	45	5	Absent	J	M/M
		270	58	7	Absent	J	J/J
	QB	350	46	0	Absent	M	M/M
		351	51	0	Absent	M	M/M
		352	43	0	Absent	M	J/J
		353	43	0	Absent	M	M/M
		355	43	0	Absent	M	J/M
		356	33	1	Absent	J	J/J
		357	52	0	Absent	M	J/M
		358	41	0	Absent	M	M/M
		359	53	0	Absent	M	M/M
		360	44	1	Absent	M	M/M
		361	54	0	Absent	M	M/M

SK	392	46	4	Absent	J	J/J
	393	41	3	Absent	J	J/J
	394	53	6	Absent	J	M/M
	395	53	5	Absent	J	J/M
	396	42	6	Absent	J	J/J
	397	30	0	Absent	J	J/J
	398	51	1	Absent	J	J/J
	399	35	3	Absent	J	J/J
	400	47	2	Absent	J	J/J
	401	53	1	Absent	J	J/J
RR	457	38	0	Present	T	T/T
	459	51	1	Absent	J	J/J
	460	51	1	Absent	J	J/J
	461	33	6	Absent	J	J/T
	462	50	4	Absent	J	J/J
	463	55	5	Absent	J	J/J
	464	54	5	Absent	J	J/J
	465	41	3	Absent	J	J/J
	466	34	3	Absent	J	J/T
	467	45	5	Absent	J	J/J
	468	49	3	Absent	J	J/J
	469	50	4	Absent	J	J/J
	470	31	2	Absent	J	J/J
	471	56	0	Present	T	T/T
MH	471	50	0	Absent	M	M/M
	472	34	0	Absent	M	M/M
	473	45	0	Absent	M	J/M
	474	52	0	Absent	M	J/J
	475	53	1	Absent	M	J/M
	476	54	0	Absent	M	M/M
	477	40	0	Absent	M	M/M
	478	38	0	Absent	M	M/M
	479	47	0	Absent	M	M/M
	480	47	0	Absent	M	M/M
	481	30	0	Absent	M	M/M
	482	48	0	Absent	M	J/J
	483	54	0	Absent	M	J/M
	484	53	1	Absent	M	J/M
	485	42	0	Absent	M	J/M
QS	486	36	1	Absent	J	J/M
	487	58	3	Absent	J	J/M
	488	52	2	Absent	J	J/J
	489	49	1	Absent	J	J/M
	490	33	5	Absent	J	J/M
	491	44	0	Absent	M	M/M
	492	41	0	Absent	J	J/J
	493	48	4	Absent	J	J/M
	494	48	4	Absent	J	J/J
	495	60	1	Absent	M	J/M
	496	43	1	Absent	J	J/M
	497	46	3	Absent	J	J/J
	498	45	2	Absent	J	J/J
	499	54	4	Absent	J	J/J
	500	36	2	Absent	J	J/M
QU	501	43	0	Absent	M	J/M
	502	45	0	Absent	M	M/M

		503	44	0	Absent	M	J/M
		504	48	0	Absent	M	M/M
		505	44	0	Absent	M	M/M
		506	55	0	Absent	M	M/M
		507	49	1	Absent	M	M/M
		508	48	0	Absent	M	M/M
		509	56	0	Absent	M	M/M
		510	58	1	Absent	M	M/M
		511	52	0	Absent	M	M/M
		512	60	0	Absent	M	M/M
		513	38	0	Absent	M	M/M
		514	33	0	Absent	M	J/M
		515	41	0	Absent	M	J/M
	HY	516	34	7	Absent	J	M/M
		517	35	1	Absent	J	J/J
		518	56	1	Absent	J	J/J
		519	32	1	Absent	J	J/J
		520	51	5	Absent	J	J/J
		521	46	2	Absent	J	J/T
		522	44	7	Absent	J	J/J
		523	37	1	Absent	J	J/M
		524	54	3	Absent	J	J/J
		525	48	1	Absent	J	J/J
	RF	642	51	8	Absent	J	J/J
		643	36	8	Absent	J	J/J
		644	42	3	Absent	J	J/J
		645	44	11	Absent	J	J/J
		646	50	11	Absent	J	J/J
		647	58	10	Absent	J	J/J
		648	59	7	Absent	J	J/J
		649	58	9	Absent	J	J/J
		650	36	10	Absent	J	J/J
		651	32	9	Absent	J	J/J
		652	57	11	Absent	J	J/J
		653	54	6	Absent	J	J/J
		654	32	5	Absent	J	J/J
	BN	655	39	9	Absent	J	J/J
		656	39	6	Absent	J	J/J
		657	55	11	Absent	J	J/J
		658	53	9	Absent	J	J/J
		659	57	11	Absent	J	J/J
		660	54	5	Absent	J	J/J
		661	59	12	Absent	J	J/J
		662	49	13	Absent	J	J/J
		663	48	7	Absent	J	J/J
		664	53	1	Absent	J	J/J
Palmer Creek	BE	598	50	0	Absent	M	M/M
		599	43	0	Absent	M	M/M
		600	54	0	Absent	M	M/M
		601	43	1	Present	M	J/J
		602	55	0	Absent	M	M/M
		603	46	0	Absent	M	M/M
		604	47	0	Absent	M	J/M
		605	46	0	Absent	M	M/M
		606	38	0	Absent	M	J/M
		607	49	0	Absent	M	M/M

	608	52	0	Absent	M	J/J
	609	49	0	Present	M	M/M
	610	53	0	Absent	M	M/M
	611	38	0	Present	T	T/T
BD	612	51	1	Absent	M	M/M
	613	51	0	Present	M	M/M
	614	49	0	Absent	M	M/M
	615	53	0	Absent	M	J/M
	616	49	1	Present	M	M/M
	618	55	0	Present	T	T/T
	619	69	0	Present	T	T/T
	620	32	0	Absent	M	J/M
	621	58	0	Present	T	T/T
	622	33	0	Present	T	J/T
	623	32	1	Absent	M	M/M
	624	61	0	Present	T	T/T
	625	63	0	Present	T	T/T
BC	626	48	1	Absent	M	M/M
	627	48	0	Absent	M	J/M
	628	55	0	Present	M	J/M
	629	42	1	Absent	M	M/M
	630	39	0	Absent	M	M/M
	631	50	1	Absent	M	J/J
	632	55	0	Absent	M	J/M
	633	53	1	Absent	M	J/J
	634	44	1	Absent	M	J/M
	635	33	0	Absent	M	J/M
	636	47	0	Absent	M	M/M
	637	57	0	Absent	M	T/T
	638	57	1	Present	M	M/M
	639	53	0	Present	T	T/T
	640	63	0	Present	T	T/T
	641	64	0	Present	T	T/T
BF	725	43	0	Absent	M	J/M
	726	44	0	Absent	M	M/M
	727	38	0	Absent	M	J/M
	728	40	0	Absent	M	M/M
	729	47	0	Absent	M	M/M
	730	55	0	Absent	M	M/M
	731	54	1	Absent	M	J/J
	732	56	0	Absent	M	J/J
	733	46	0	Absent	M	J/M
	734	55	0	Absent	M	J/M
	735	48	3	Absent	M	J/M
	736	40	1	Absent	M	J/M
	737	55	0	Absent	M	M/M
BJ	738	57	1	Absent	M	M/M
	739	48	0	Absent	M	J/M
	740	40	1	Absent	M	J/M
	741	38	0	Absent	M	M/M
	742	66	0	Present	T	T/T
BK	743	52	0	Present	T	T/T
	744	60	0	Present	T	T/T
	745	61	0	Present	T	T/T
	746	58	0	Present	T	T/T
	747	69	1	Present	T	T/T

		748	54	0	Present	T	T/T
		749	70	0	Present	T	T/T
		750	72	0	Present	T	T/T
Mt Sterling	ZI	752	46	2	Present	J	J/T
		753	48	8	Absent	J	J/J
		754	56	5	Absent	J	J/J
		755	50	5	Absent	J	J/J
		756	36	3	Present	J	J/J
		757	38	6	Absent	J	J/J
		758	49	10	Absent	J	J/J
		762	52	6	Absent	J	J/J
		763	35	3	Absent	J	J/J
		764	42	1	Absent	M	J/J
		765	46	8	Absent	M	J/J
		766	49	5	Absent	M	J/J
	ZB	767	65	0	Present	T	T/T
		768	52	0	Present	T	T/T
		769	51	0	Present	T	T/T
		770	76	0	Present	T	T/T
		771	35	0	Present	T	T/T
		772	53	0	Present	T	T/T
		774	65	0	Present	T	T/T
		777	36	0	Present	T	T/T
		778	35	0	Present	T	T/T
		779	57	0	Present	T	T/T
	ZE	780	59	2	Absent	T	J/J
		781	63	0	Present	T	J/T
		782	56	0	Present	M	J/J
		783	55	1	Present	M	T/T
		784	43	0	Absent	T	J/J
		785	54	0	Present	M	J/T
		786	43	4	Present	T	J/J
		787	57	5	Absent	M	J/T
		788	55	0	Present	T	J/J
		789	58	2	Absent	T	J/J
		790	44	7	Present	M	J/T
	ZD	791	49	0	Present	T	T/T
		792	62	1	Absent	M	J/J
		794	51	0	Present	T	T/T
		795	49	1	Present	T	J/T
		796	52	0	Present	T	T/T
		797	59	0	Present	T	T/T
		798	40	0	Present	T	T/T
		799	48	0	Present	T	T/T
		800	42	0	Present	T	T/T
		801	63	0	Present	T	T/T
		802	64	0	Present	M	J/T
	ZL	951	39	6	Absent	J	J/J
		952	34	5	Absent	J	J/J
		953	36	7	Absent	J	J/J
		954	52	6	Absent	M	J/J
		955	50	4	Absent	J	J/J
		956	57	9	Absent	J	J/T
		957	53	3	Absent	M	J/J
		958	52	2	Absent	J	J/J
		959	54	8	Absent	J	J/J

	960	50	7	Absent	M	J/J
	961	52	3	Absent	J	J/J
	962	45	8	Absent	M	J/J
ZM	963	43	4	Absent	T	J/J
	964	53	4	Absent	J	J/J
	965	48	4	Absent	J	J/J
	966	57	8	Absent	J	J/J
	967	58	9	Absent	J	J/T
	968	54	7	Absent	J	J/J
	969	44	6	Absent	J	J/J
	970	53	10	Absent	J	J/J
	971	44	4	Absent	J	J/J
	972	46	11	Absent	J	J/J
ZK	973	47	8	Absent	M	J/J
	974	53	8	Absent	J	J/J
	975	56	7	Absent	J	J/J
	976	53	1	Absent	J	J/J
	977	53	6	Absent	M	J/J
	978	38	7	Absent	M	J/J
	979	59	8	Absent	M	J/J
	980	53	2	Absent	J	J/J
	981	44	10	Absent	J	J/J
	982	50	3	Absent	J	J/J
	983	48	5	Absent	J	J/J
	984	47	6	Absent	M	J/J
ZJ	985	55	3	Absent	J	J/J
	987	48	3	Absent	J	J/T
	988	48	2	Absent	J	J/T
	989	60	5	Absent	M	J/J
	990	50	1	Absent	J	J/J
	991	48	0	Absent	J	J/J
	992	54	7	Absent	M	J/J
	993	55	1	Absent	J	J/J
	994	52	8	Absent	J	J/J
	995	42	3	Absent	J	J/J
	996	55	5	Absent	J	J/J
ZH	997	56	2	Absent	M	J/J
	998	54	0	Absent	M	J/J
	999	63	6	Absent	M	J/J
	1000	55	8	Absent	M	J/J
	1001	57	1	Absent	M	J/J
	1002	62	2	Absent	M	J/J
	1003	60	3	Absent	M	J/J
	1004	60	5	Absent	M	J/J
	1005	61	8	Absent	M	J/J
	1006	48	3	Absent	M	J/J
	1007	40	1	Absent	M	J/J
	1008	34	7	Absent	M	J/T
ZF	1009	55	8	Absent	M	J/J
	1010	44	5	Absent	M	J/J
	1011	51	4	Absent	M	J/J
	1012	56	2	Absent	M	J/T
	1013	48	6	Absent	M	J/J
	1014	51	5	Absent	M	J/J
	1015	50	6	Absent	T	T/T
	1016	54	1	Absent	M	J/J

	1017	52	3	Absent	M	J/M
	1018	45	6	Absent	M	J/J
	1019	38	3	Absent	M	J/J
	1020	41	9	Absent	M	J/M
ZG	1021	57	4	Absent	M	J/J
	1022	51	6	Absent	M	J/J
	1023	52	3	Absent	M	J/T
	1024	53	4	Absent	M	J/J
	1025	54	0	Absent	M	J/J
	1026	41	7	Absent	M	J/J
	1027	44	0	Absent	M	J/J
	1028	38	8	Absent	M	J/J
	1029	39	5	Absent	M	J/J
	1030	55	9	Absent	M	J/J
ZC	1031	60	7	Absent	T	J/T
	1032	49	4	Present	T	J/T
	1033	37	3	Absent	T	J/J
	1034	36	5	Present	T	J/J
	1035	36	1	Absent	M	T/T
	1036	35	0	Present	T	J/T
	1037	35	5	Absent	T	J/J
	1038	36	2	Absent	M	J/T
	1039	50	0	Absent	M	J/T
	1040	59	5	Absent	T	J/T
	1041	64	1	Absent	T	J/J
	1042	55	0	Present	T	J/J
ZA	1043	37	0	Present	T	T/T
	1046	55	0	Present	T	T/T
	1047	69	0	Present	T	T/T
	1048	64	0	Present	T	T/T
	1049	57	0	Present	T	T/T

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[1] See Figure 1 and Appendix 1 for site locations.
[2] Individual codes are those assigned by the authors.
[3] SVL (Snout Vent Length) was measured from the tip of the snout to the posterior end of the cloaca.
[4] Cheek pigmentation was scored on a scale of 0 to 13, with a score of 0 indicating a complete lack of red pigment and 13 indicating extensive and bright red pigment.
[5] As the mitochondrial genome is haploid, only a single haplotype copy is given.
[6] As the nuclear genome is diploid, two haplotypes are given.

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CHAPTER IV

Past, present, and future range dynamics of the montane salamanders *Plethodon jordani* and *P. metcalfi* (Caudata: Plethodontidae)

Abstract

In this study, the range dynamics of two species of montane southern Appalachian salamanders, *Plethodon jordani* and *P. metcalfi*, are examined using ecological niche modeling (ENM) at three time periods: (1) present-day, (2) during the last glacial maximum 21,000 years ago, and (3) in the year 2050 under a climate warming scenario. Models generated using present-day bioclimatic variables indicate that *P. jordani* and *P. metcalfi* currently occupy nearly all of the regions that are predicted to contain suitable habitat. Models that reconstruct distributions during the last glacial maximum show little change from the present-day, although the resolution at which these models were created may be an issue. Future projections indicate that the ranges of *P. jordani* and *P. metcalfi* will be substantially smaller than present-day as the species migrate upslope to track shifts in suitable habitat. The conservation status of *P. jordani* and *P. metcalfi* are discussed in light of modern climate change.

Introduction

Understanding historic and present-day geographic distributions of species are important for inferring evolutionary processes (Highton and Peabody 2000, Weisrock and

Larson 2006, Rodríguez-Sánchez and Arroyo 2008). Information on species' ranges can give insight into the mode of speciation (e.g., sympatric versus allopatric; Barraclough and Vogler 2000), reveal patterns of historic gene flow that may help to explain present phylogenetic relationships (e.g., Crespi et al. 2003), or aid in the testing of other evolutionary hypotheses (e.g., selection versus drift as a cause of divergence in mountain-top isolates; Knowles and Richards 2005). Furthermore, there are an increasing number of studies that examine range shifts in response to past climatic oscillations in the Pleistocene (Davis and Shaw 2001, Peterson et al. 2004, Brito 2007). For example, the last glacial maximum, which occurred about 21,000 years ago, fragmented many European and North American species into low latitude refugia (Hewitt 1996, 2000, Davis and Shaw 2001, Waltari et al. 2007).

Species inhabiting mountainous areas are predicted to be differentially affected by climate change (Hewitt 1996, Guralnick 2007, but see Peterson 2003). Under a cooling climate scenario, rather than migrate northwards as predicted for flatland species, montane species will migrate downslope to track the movement of suitable habitat. Conversely, in a warming climate, ranges are predicted to shift upslope. This latter prediction has many important consequences: First, ranges will necessarily contract as the conical shape of mountains means there is less area at higher elevations (Peterson 2003). Second, populations will become fragmented into mountain-top isolates, often with little or no gene flow between them (e.g., McDonald and Brown 1992, Highton and Peabody 2000). Lastly, if warming persists or intensifies, species may go extinct (McDonald and Brown 1992, Peterson et al. 2002).

Shifting ranges in response to climate change may also lead to differences in hybrid zone dynamics. Hybrid zone movement as a result of climate change has not been empirically well-documented. One rare example is from a ground cricket hybrid zone (*Allenomobius*) in the Appalachian Mountains (Britch et al. 2001). In that study, researchers document an upslope shift in hybrid zone position that they credit to regional warming trends. It should be noted, however, that definitive evidence is lacking. There is a growing body of empirical evidence that habitat disturbances other than those caused by climate change may result in shifts in hybrid zone position. For example, in a 20-year morphological study, Hairston et al. (1992) documented an upslope shift in the position of a hybrid zone between two species of salamanders in the genus *Plethodon*. The authors correlate the shift with the cessation of logging activity near the beginning of the last century. Similarly, the overgrazing of cattle may have altered the relative abundance of preferred habitats of two species of fence lizards (*Sceloporous*) in the southwestern United States (Leaché and Cole 2007). The change in habitat may have resulted in what appears to be a 1.5 km northward shift of the hybrid zone over a 30-year period. Lastly, conversion of land for agriculture appears to have favored one species of South American *Pholidobolus* lizard over a congener with which it hybridizes (Hillis and Simmons 1986). The shift in hybrid zone position appears to have taken place rapidly, within a 12-year period. The above examples demonstrate the sensitivity of hybrid zones to environmental perturbations, be they anthropogenic or natural.

Modern climate change is occurring at an unprecedented rate (Schellnhuber 2008). Estimates place the anticipated global temperature increase during the 21st century at 1.4-4.3^{o}C, with a reasonable expectation of 2.4^{o}C (Ramanathan and Feng 2008).

Furthermore, new research also suggests atmospheric carbon dioxide levels will not decline for at least 1,000 years after emissions stop (Solomon et al. 2008). The number of documented range shifts occurring during recent warming trends is growing (Root et al. 2003), as is the diversity of taxa involved (butterflies, Parmesan et al. 1999; crickets, Britch et al. 2001; amphibians and reptiles, Raxworthy et al. 2008; birds, Thomas and Lennon 1999; mammals, Moritz et al. 2008). Anticipated effects of modern climate change resulting from increased temperatures, greater temperature variability, and seasonal precipitation changes are likely to be severe for amphibian species (Daszak et al. 2005, McMenamin et al. 2008). Furthermore, studies suggest that the generally low dispersal abilities of amphibians make them particularly susceptible to the effects of climate change (Araújo and Pearson 2005, Araújo et al. 2006).

Increasingly, evolutionary biologists are utilizing ecological niche modeling (ENM), also called habitat distribution modeling, to examine range dynamics (Kozak et al. 2008, Swenson 2008). ENM is a GIS-based method for predicting species distributions given georeferenced collection localities and regional environmental data. Typical environmental variables, termed bioclimatic variables, are biologically relevant and usually involve measures of temperature (e.g., maximum temperature of warmest month) and precipitation (e.g., precipitation of driest month). Bioclimatic variables are spatially interpolated from climate stations and are used to create GIS layers known as “climate surfaces” (Hijmans et al. 2005). When used in conjunction with locality information, these layers provide an estimate of a species realized ecological niche (Phillips et al. 2006). Potential distributions, as predicted by regional bioclimatic variables, are an estimate of suitable niche space that, depending upon further sampling, may or may not be occupied by the species in question. Reasons why potential niche space is not occupied include human influences (e.g., habitat degradation), biotic interactions (e.g., competition from other species), and geographic barriers (Pearson and Dawson 2003, Phillips et al. 2006). Additionally, insufficient dispersal time may also explain why potential niche space is not occupied (Araújo and Pearson 2005).

Recently, it has become possible to create niche models based on paleoclimatic data and inferred future climate scenarios. When modeling is performed at glacial maxima, it is possible to infer past range expansions (Rodríguez-Sánchez and Arroyo 2008). Such data have proved useful when testing hypotheses of range-shift dynamics after secondary contact in hybrid zones. For example, Cicero (2004) documented discordance between the position of an avian hybrid zone as determined using morphological and molecular markers and a change in vegetation community as evidenced through ENM. Increasingly, researchers are using ENM to examine the effects of modern climate change in the dynamics of species ranges under anticipated climate change scenarios (Peterson et al. 2002, Parra-Olea et al. 2005).

Plethodon jordani and *P. metcalfi* (family Plethodontidae) are high-elevation salamander species found in the southern Appalachians. The present-day range of *P. jordani* is restricted to the Great Smoky Mountains and the range of *P. metcalfi* is restricted to the Blue Ridge and Balsam Mountains (see Figure 4.1A). In a morphological study of hybrid zone dynamics between the two salamander species, Hairston et al. (1992) presented a hypothesis on the historic ranges of the species that accounts for the present day pattern of hybrid formation. Using the morphological trait of red-cheek pigmentation, which is found in *P. jordani* but is absent in *P. metcalfi*, the authors mapped the frequency of hybrids along a transect through the hybrid zone. The transition was found to be simple and sigmoidal. In a model assuming neutral diffusion of the measured trait, the authors concluded that isolation between the species last occurred about 5,000 years ago during the Hypsithermal Interval, a period characterized by warmer temperatures than those found today. During that time, ranges would have contracted and *P. jordani* and *P. metcalfi* would likely have been isolated from one another.

There are two goals of this study. First, to reconstruct the historic range fluctuations of *P. jordani* and *P. metcalfi* by modeling the species' ranges during the last glacial maximum 21,000 years ago and present-day. Temperatures during the last glacial maximum were colder and, consequently, distributions are predicted to be greater than at present. Second, to make predictions using ENM on the future ranges of *P. jordani* and *P. metcalfi* under the specter of modern climate change.

Methods

Sampling for ecological niche modeling

Locality information used in the ecological niche models is a subset of that used by Kozak and Wiens (2006). Georeferenced localities for *Plethodon metcalfi* (n = 289) and *P. jordani* (n = 374) were obtained from the collection of the U. S. National Museum. Most specimens used in the analysis were collected by R. Highton and were assigned to species using morphology and allozymes. Any species with uncertain identification was omitted from the analysis (Kozak and Wiens 2006).

Constructing ecological niche models

Models were created using Maxent version 3.2.1 (Phillips et al. 2004, Phillips et al. 2006). Maxent is a machine-learning method which utilizes presence-only data in the construction of models of species distributions. Like other ENM methods, the objective of Maxent is to predict environmental suitability within a specified area given the environmental conditions of known collection localities of a population or species of interest. Maxent does this by creating a probability distribution of habitat suitability for all grid cells in the specified region by computing the probability distribution of maximum entropy, i.e., a distribution that is closest to uniform with respect to the average of environmental conditions at the known collection localities. Habitat suitability values for each grid cell are given as a probability, and probability values for all grid cells must sum to 1.0. Each cumulative probability value is subsequently multiplied by 100 to give a percentage. Raster files of habitat suitability values created in Maxent were converted to ASCII files and viewed in ArcGIS version 9.3.

The bioclimatic variables used to model species distributions consisted of the 19 available from the WorldClim database (www.worldclim.org; Table 4.1). Many ENM studies attempted to minimize non-independence among variables by including representatives from groups of highly correlated variables. Maxent differs from many other niche modeling programs in that it assesses and incorporates both variables and their interactions, thus accounting for non-independence of variables (Phillips et al. 2006).

There is considerable inherent uncertainty when inferring past and present climate scenarios. Consequently, the resolution at which models were made differs between the present-day and those from the past and future. Present-day models were created at a 30 arc-second resolution, which equates to a grid cell size of approximately 1 km^2 (Hijmans et al. 2005). Models for the last glacial maximum 21,000 years ago and future projections for the year 2050 were created at 2.5 arc-minute resolution, which equates to a grid cell size of approximately 25 km^2. It should be noted that models using climate projections for the year 2050 are based on Global Climate Models (GCMs; also called General Circulation Models) and are dependent upon assumptions of future carbon emissions and their effects (www.worldclim.org). Furthermore, predicting climate patterns has a large stochastic component that may result in differences between observed and actual climate scenarios (www.worldclim.org).

Results

Range-wide predictions of the ecological niche models

Ecological niche models generated using present-day bioclimatic variables indicate that *Plethodon jordani* and *P. metcalfi* currently occupy nearly all of the regions that are predicted to contain suitable habitat. The present distribution of *P. jordani* is restricted to the Great Smoky Mountains, and habitat in that region is predicted to be highly suitable (Figure 4.1B). Portions of the Balsam Mountains, which are contiguous with the Great Smoky Mountains (Figure 4.1A), are also predicted to be inhabited by *P. jordani* with high suitability. The actual present-day distribution of *P. metcalfi* is the Balsam and Blue Ridge Mountains, and the predicted distribution is largely concordant with the species’ actual range (Figure 4.1C). According to the models, the southeastern portions of the Great Smoky Mountains are also highly suitable habitat for *P. metcalfi*, although the distribution of this habitat is somewhat patchy. Disjunct mountain ranges to the southwest and northeast are also predicted to contain suitable habitat for both species.

Out of the 19 bioclimatic variables used to construct the ecological niche models, two highly correlated variables emerged as important (Table 4.1). BIO14, precipitation in the driest month, and BIO17, precipitation in the driest quarter, together explain 77.4% of the predicted distribution for *P. jordani* and 84.7% for *P. metcalfi*. A third variable, precipitation seasonality (BIO15), explains a further 6.9% of the distribution of *P. jordani* and 7.9% of the distribution for *P. metcalfi*.

Projected habitat suitability values during the last glacial maximum were markedly lower throughout much of the southern Appalachians (note change in suitability values associated with colors in Figures 4.2A and B). Highly suitable habitat (arbitrarily set at 80-100%) is absent for either species during the last glacial maximum. In addition, the suitability values for *P. metcalfi* tend to be lower throughout the region (maximum of 48%) that those for *P. jordani* (maximum of 80%). Overall, ranges are not expected to have shifted greatly for either *P. jordani* or *P. metcalfi*.

Both *P. jordani* and *P. metcalfi* in the year 2050, under the scenario of climate warming, show a dramatic reduction is suitable habitat. This results from reduced habitat quality (i.e., there is a reduction in the maximum habitat suitability value) and reduced geographic extent of suitable habitat (Figures 4.3A and B). Habitat suitability values are substantially lower than those predicted in present-day models, with the maximum values predicted for *P. jordani* and *P. metcalfi* being 36% and 29%, respectively. The highest available habitat for both species in the year 2050 is restricted to the highest elevations throughout the southern Appalachians.

Discussion

Historical range shifts of Plethodon jordani *and* P. metcalfi

Based on the ecological niche modeling results, both *Plethodon jordani* and *P. metcalfi* currently occupy a large portion of their predicted distributions. Exceptions include mountain ranges to the southwest (e.g., the Nantahala and Unicoi Mountains) and northeast (e.g., the Newfound Mountains) (Figure 4.1A), where there are similarities in the high-elevation vegetation communities (Hairston 1949, Little 1970) and climate. Furthermore, the absence of *P. jordani* and *P. metcalfi* from these neighboring mountain ranges is expected given the valleys of unsuitable habitat separating them and the presence of other members of the *P. jordani* species complex currently found there (Highton and Peabody 2000). Additionally, the models suggest that *P. jordani* and *P. metcalfi* have different habitat requirements, as the predicted distributions of the two species only minimally overlap. In general, *P. jordani* is predicted to occur in higher elevations in the northwest regions of the southern Appalachians, while *P. metcalfi* is predicted to occur throughout many of the southwestern mountains ranges. This result is expected since the bioclimatic variables determined to be the best indicators of habitat suitability are both measures of precipitation during the driest parts of the year (Table 4.1). Presumably, much of the range of *P. metcalfi* falls within the rain shadow of the high-elevation Great Smoky Mountains. Also highlighted by the ENM results is the tolerance of *P. metcalfi* to a greater range of environmental conditions. Individual *P. metcalfi* were sampled in areas with a predicted habitat suitability value as low as 15% (known as the habitat suitability threshold), whereas the threshold for *P. jordani* is 33%.

Ecological niche models for the last glacial maximum show a reduction in maximum suitable habitat, but little change in area. Reconstructions of vegetation communities using pollen and macrofossil data show that much of the southeastern United States was cooler and dominated by cool-adapted vegetation communities during the last glacial maximum (Watts 1980, Davis 1983, Jackson et al. 2000). Thus, the amount of suitable habitat available for montane *Plethodon*, which are found almost exclusively in cool-adapted vegetation communities (spruce-fir, cove hardwood, and northern hardwood), was almost certainly much greater than at present. Reconciling these results may lie in the inherent limitations of model resolution (see below).

The effects of modern climate change

Species inhabiting mountainous areas are expected to experience range shifts upslope to track parallel shifts in suitable habitat (McDonald and Brown 1992, Hill et al. 2002, Rull and Vegas-Vilarrúbia 2006, Guralnick 2007, Raxworthy et al. 2008, but see Peterson 2003). This pattern is especially likely for eastern *Plethodon,* as previous work suggests many species within this group have experienced niche conservatism (Kozak and Wiens 2006) during past range fluctuations. Furthermore, a recent laboratory study on another genus of plethodontid salamander (*Desmognathus*) suggests these salamanders are near their physiological climate limit (Bernardo and Spotila 2006). It seems probable, therefore, that montane plethodontid salamanders are extremely vulnerable to the adverse effects of climate change.

Hairston et al. (1992) suggested that warm climates during the Hypsithermal Interval 5,000 years ago resulted in range contractions of *P. jordani* and *P. metcalfi* and, consequently, the separation of their ranges. At a broad scale, the ecological niche models presented here for the year 2050 show that both *P. jordani* and *P. metcalfi* may experience severe range contractions. This is especially true for *P. jordani,* as the range of suitable habitats appears to be narrower and restricted to the highest elevations. However, the models also show increased overlap of the most suitable habitat for each species. Thus, the area of contact between the species, as well as the geographic extent of hybridization, may increase. Furthermore, recent work suggests that the hybrid zone occurring between *P. jordani* and *P. metcalfi* may be moving southwards into the present-day range of *P. metcalfi* (see Chapter III). Models presented here show that habitat suitability values for *P. jordani* in the Great Smoky and Balsam Mountains may be similar in the year 2050 (29-36%; Figure 4.3A), which may lead to the continued southward movement of the hybrid zone.

Increasingly, studies are documenting amphibian declines as a result of climate change (Araújo et al. 2006, Pounds et al. 2006, Laurance 2008, McMenamin et al. 2008, Wake and Vredenberg 2008). Montane plethodontid salamanders in the new world tropics seem especially affected (Parra-Olea et al. 1999). Parra-Olea et al. (2005) constructed ecological niche models for two Middle American montane plethodontid salamanders, and concluded that the species could lose up to 75% of their habitat by 2050 as a result of climate change.

The current status of *P. jordani* and *P. metcalfi* populations is unknown. Only two studies directly address declines in eastern *Plethodon* and neither use rigorous tests, instead relying on observational data collected for other purposes. Furthermore, their conclusions are strongly at odds with one another. Hairston and Wiley (1993) made annual collections of *P. jordani*, *P. shermani*, and *P. teyahalee* along two elevational transects. Their collections were part of a series of class field trips and few attempts were made to standardize search time, search area, or collector enthusiasm. Their results suggest considerable fluctuation from year to year, but no trend of declining populations. In a more comprehensive study, Highton (2005) made observations at 127 localities representing 38 of the 45 species of eastern *Plethodon* over a 35-year time period. As in Hairston and Wiley (1993), the author made little attempt to standardize methodology. The results indicate no decline from the early 1950s to the mid-1980s, followed by a severe decline of 58.4% from the late 1980s to the 1990s. Importantly, every species sampled (including *P. jordani* and *P. metcalfi*) experienced declines.

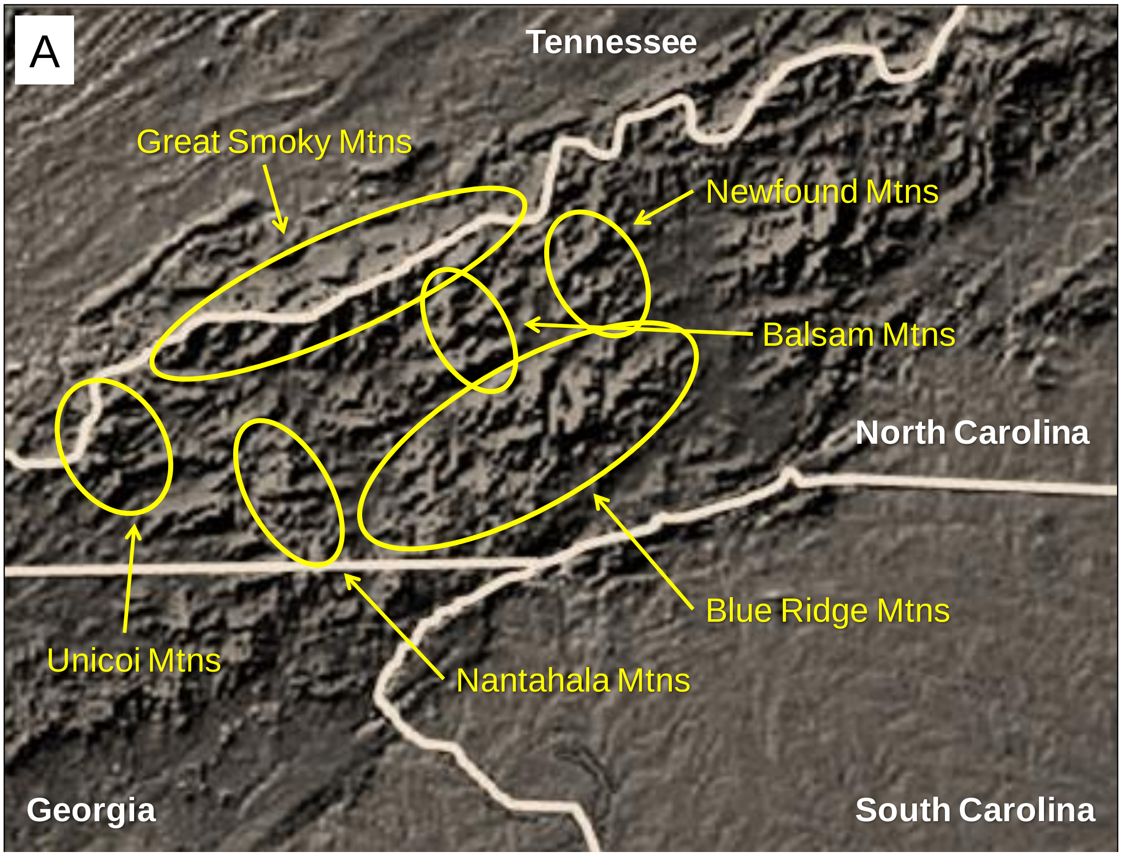
Conclusion

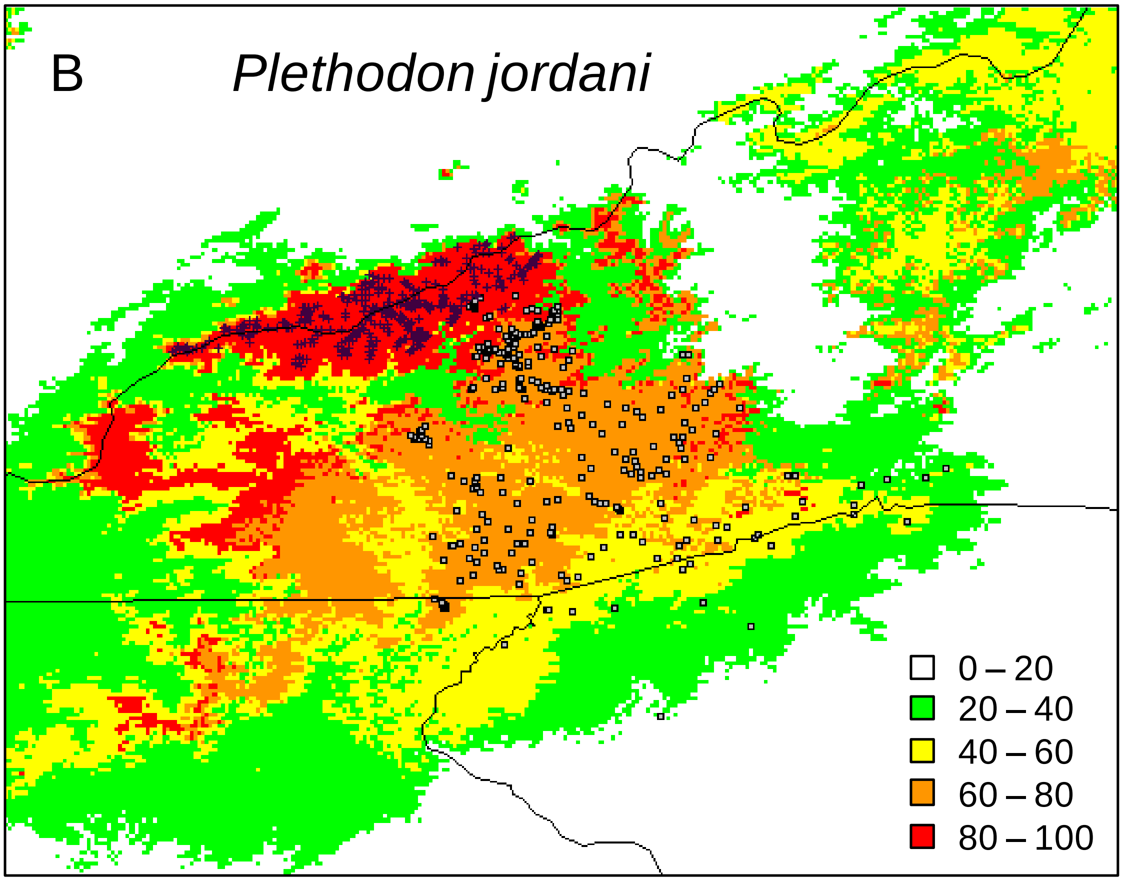
Predicted distributions of *P. jordani* and *P. metcalfi* are presented for three time periods: (1) the last glacial maximum 21,000 years ago, (2) the present-day, and (3) the year 2050. The historic and future range fluctuations modeled here likely reflect the most severe climatic conditions that these species would experience. It should be emphasized, however, that an ecological niche models represents an estimate of a species' realized niche, and not its fundamental niche (Phillips et al. 2006). As such, the models presented here must be taken as an approximation of actual distributions. Furthermore, inferring historic and predicting future distributions is saddled with inherent uncertainties that may also influence modeled distributions.

Many researchers have criticized ENM approaches because of the sole reliance on climatic data in predicting species' distributions. Critics cite the known importance of biotic interactions and argue that range limits may be determined through competition with neighboring species rather than by climatically unsuitable habitat (Davis et al. 1998, Pearson and Dawson 2003, Martínez-Meyer 2005). Previous studies have suggested competitive interactions may be important in determining the distributions of *P. jordani* and *P. teyahalee* (Hairston 1980a, 1980b, 1983, Nishikawa 1985, 1987, Hairston et al. 1987, Adams 2004). Furthermore, there is some evidence that hybrids may be under negative selection. In follow-up studies to Hairston's (1980a) reciprocal transplant experiments, Highton (1998) demonstrated a deficit of heterozygotes suggesting the possibility of either selection against hybrids or assortative mating. The latter hypothesis garnered support from laboratory studies involving heterospecific crosses between *P. jordani* and *P. metcalfi* (Reagan 1992), but lowered heterozygote fitness remains a possibility. Therefore, biotic interactions, either through competition or hybridization, may limit species boundaries. If this is the case, then models may underestimate these species' potential distributions.

One major limitation of the use of ENM in this study is the resolution at which the models were created (Swenson 2008). This is true for both the 30 arc-second and 2.5 arc-minute models, in which habitat heterogeneity is measured in kilometers. This coarse resolution may obscure differences in microhabitat. If this is the case, then pockets of suitable habitat (e.g., moist ravines) may act as refugia, resulting in an underrepresentation of species distributions. This is especially true in this study, as home range size estimates for these species are small [*P. jordani*: 1.9-11.5 m^2 (Merchant 1972, Nishikawa 1990); *P. metcalfi*: 11.8-51.2 m^2 (Madison and Shoop 1970)]. Additionally, interpolation of climate surfaces in mountainous areas is problematic because of the extreme topographic variation (Hijmans et al. 2005), although this may be somewhat offset by the greater density of climate stations in the eastern United States (Hijmans et al. 2005).

Figure 4.1. Topographical relief map of southern Appalachians highlighting mountain ranges mentioned in text (A). Ecological niche modeling results showing present-day predicted geographic distribution for *Plethodon jordani* (B) and *P. metcalfi* (C). For comparison, collection localities of both *P. jordani* (+) and *P. metcalfi* (□) are shown in both maps. Colors in (B) and (C) indicate habitat suitability values as assigned by Maxent, and are given as percentages (cumulative probability x 100; see text for more detail).


A
Tennessee
Great Smoky Mtns
Newfound Mtns
Balsam Mtns
North Carolina
Unicoi Mtns
Blue Ridge Mtns
Nantahala Mtns
Georgia
South Carolina


B
Plethodon jordani
0 – 20
20 – 40
40 – 60
60 – 80
80 – 100

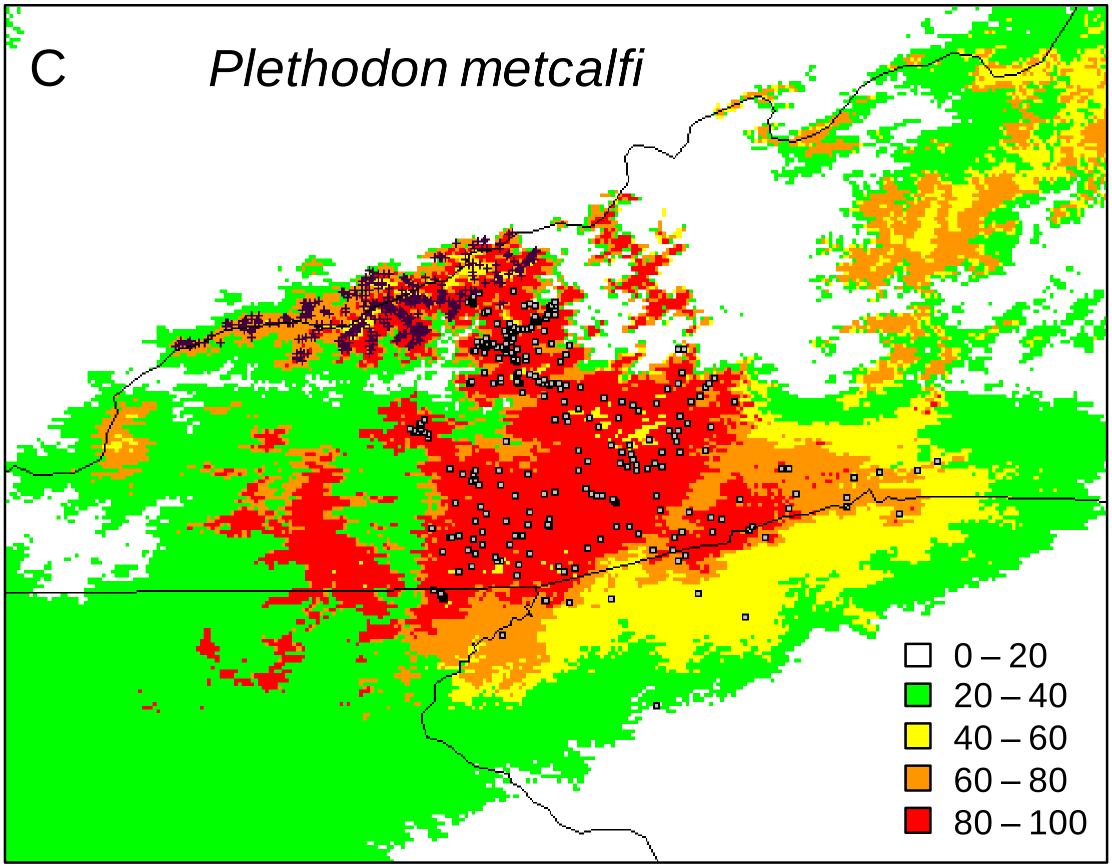
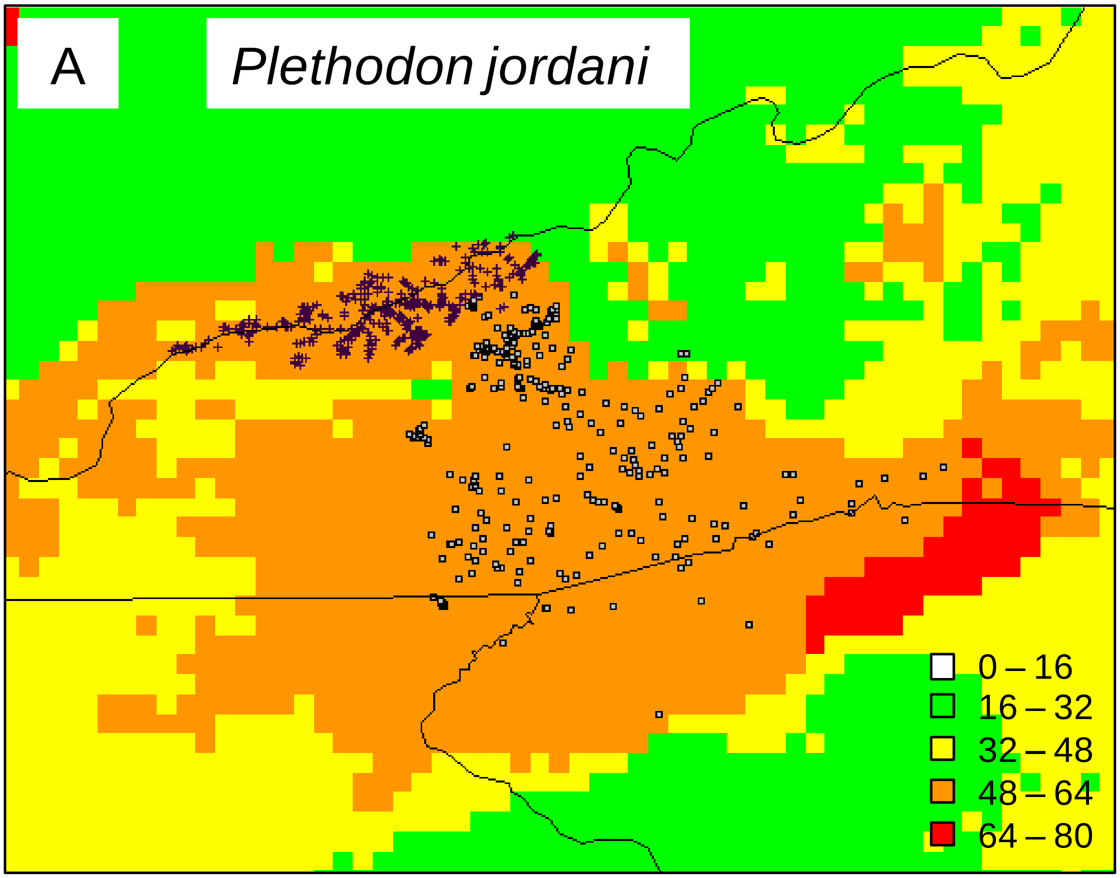

C
Plethodon metcalfi
0 – 20
20 – 40
40 – 60
60 – 80
80 – 100

Figure 4.2. Ecological niche modeling result showing predicted geographic distribution for *Plethodon jordani* (A) and *P. metcalfi* (B) during the last glacial maximum 21,000 years ago. For comparison, collection localities for both *P. jordani* (+) and *P. metcalfi* (□) are shown in both maps. Colors in (A) and (B) indicate habitat suitability values as assigned by Maxent, and are given as percentages (cumulative probability x 100; see text for more detail).


A
Plethodon jordani
0 – 16
16 – 32
32 – 48
48 – 64
64 – 80

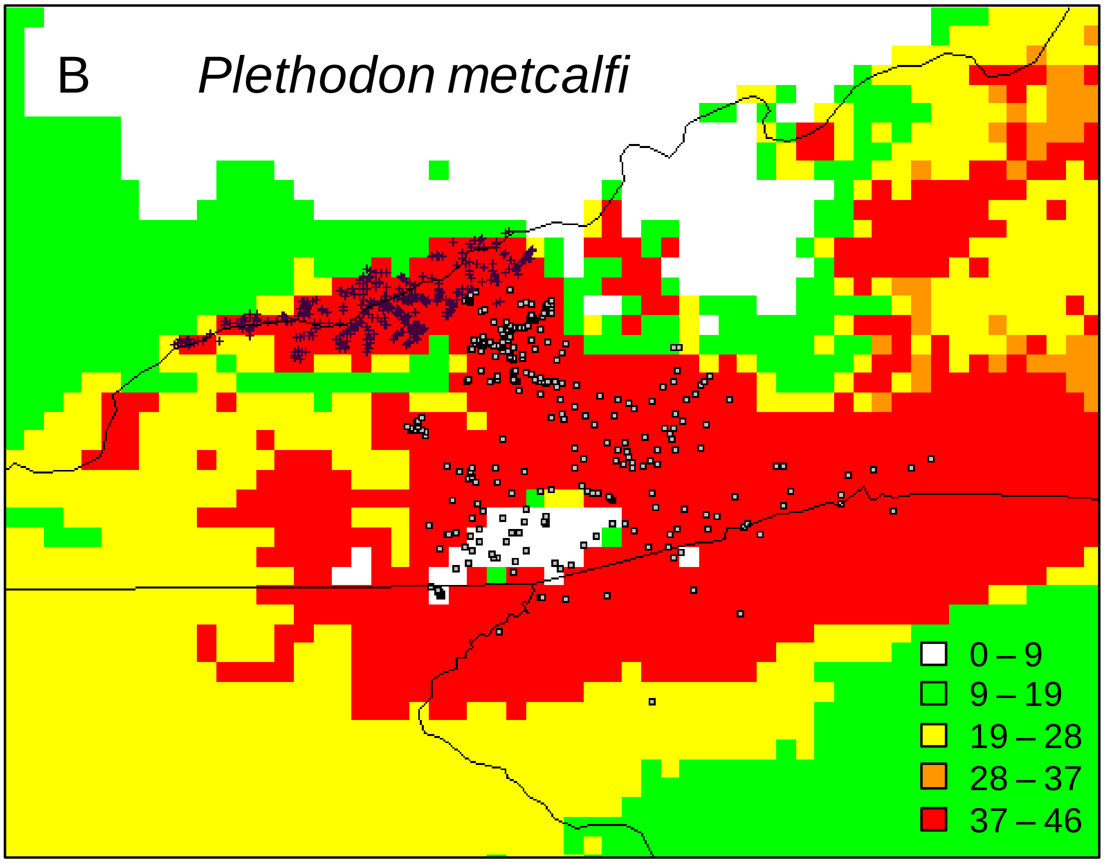
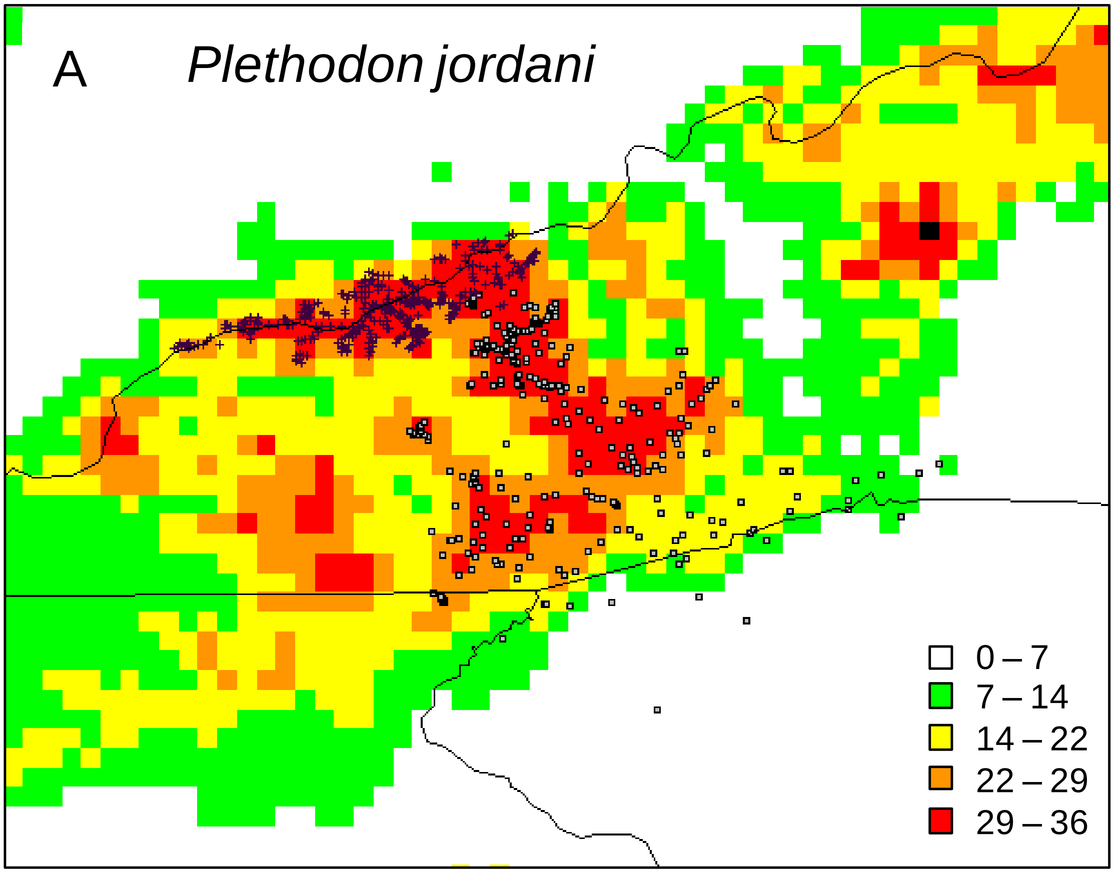

B
Plethodon metcalfi
0 – 9
9 – 19
19 – 28
28 – 37
37 – 46

Figure 4.3. Ecological niche modeling result showing predicted geographic distribution for *Plethodon jordani* (A) and *P. metcalfi* (B) in the year 2050. For comparison, collection localities for both *P. jordani* (+) and *P. metcalfi* (□) are shown in both maps. Colors in (A) and (B) indicate habitat suitability values as assigned by Maxent, and are given as percentages (cumulative probability x 100; see text for more detail).


A
Plethodon jordani
0 – 7
7 – 14
14 – 22
22 – 29
29 – 36

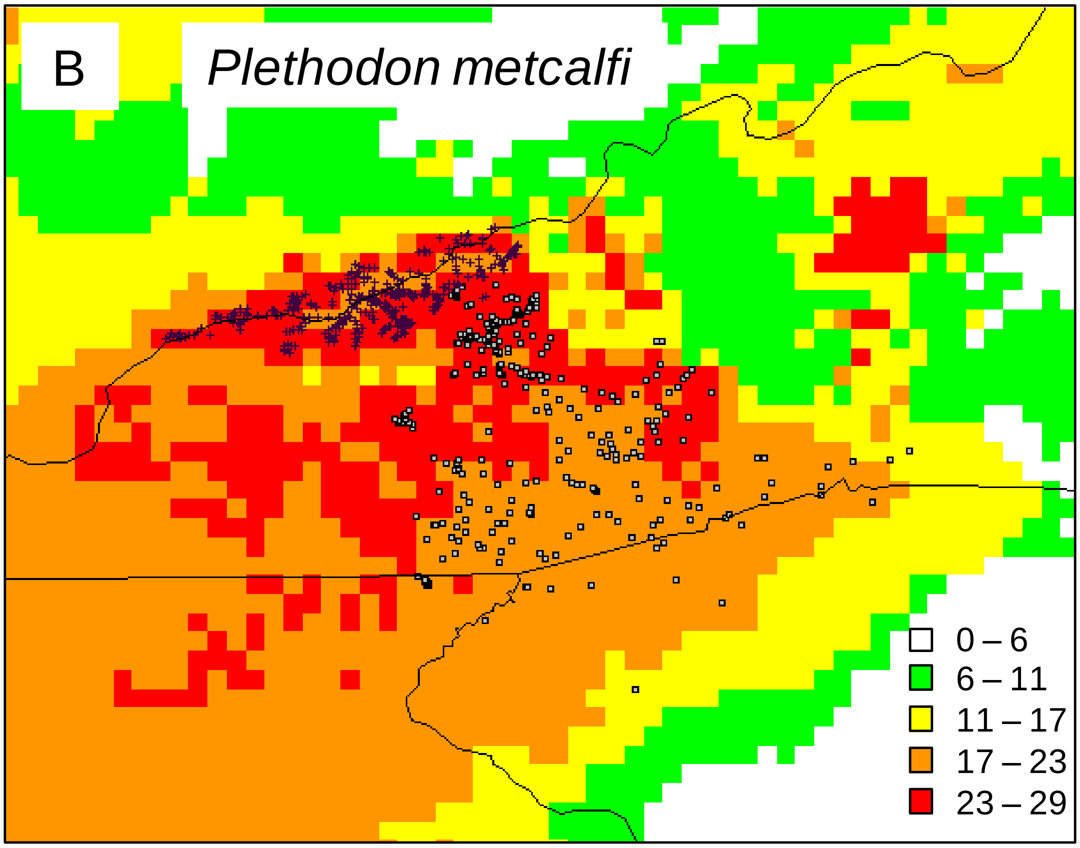

B
Plethodon metcalfi
0 – 6
6 – 11
11 – 17
17 – 23
23 – 29

Table 4.1. Climatic variables[1] used in creation of ecological niche models.

Variables[2]	Description	Percent for *P. jordani*[3]	Percent for *P. metcalfi*[3]
BIO1	Annual Mean Temperature	3.3	3.5
BIO2	Mean Diurnal Range	0.2	0
BIO3	Isothermality	0	0.1
BIO4	Temperature Seasonality	1.1	1
BIO5	Max Temperature of Warmest Month	0	0
BIO6	Min Temperature of Coldest Month	0	0
BIO7	Temperature Annual Range	0.1	0
BIO8	Mean Temperature of Wettest Quarter	2.1	0.2
BIO9	Mean Temperature of Driest Quarter	0	0.5
BIO10	Mean Temperature of Warmest Quarter	2	1.5
BIO11	Mean Temperature of Coldest Quarter	0.2	0
BIO12	Annual Precipitation	0.6	0.2
BIO13	Precipitation of Wettest Month	0.2	0.1
BIO14	Precipitation of Driest Month	19.4	53.4
BIO15	Precipitation Seasonality	6.9	7.9
BIO16	Precipitation of Wettest Quarter	0	0
BIO17	Precipitation of Driest Quarter	58	31.3
BIO18	Precipitation of Warmest Quarter	5.4	0
BIO19	Precipitation of Coldest Quarter	0.3	0.5

[1] Variables are as given on the WorldClim database, available at www.worldclim.org/bioclim.htm.

[2] Variable names are as assigned by WorldClim.

[3] Given as a percent contribution for model predictions for *Plethodon jordani* and *P. metcalfi*.

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CHAPTER V

Conclusion

The research presented in the previous chapters deals with multiple aspects of the evolutionary dynamics of species in the *Plethodon glutinosus* group, with an emphasis on three species – *P. jordani*, *P. metcalfi*, and *P. teyahalee*. There have been numerous studies on the evolution of salamanders in the family Plethodontidae and the *P. glutinosus* group in particular, leading some authors to suggest that the family is a model system for understanding evolutionary processes (Arnold et al. 1993). For those students of evolution interested in speciation and the maintenance of species boundaries, the species richness, numerous instances of hybridization, and considerable wealth of information on the behavior, ecology, and life history of most species makes plethodontid salamanders an ideal system. A central goal of this dissertation is to bridge the gap between the pioneering studies of early researchers (especially Nelson Hairston, Sr and Richard Highton) and the next generation of evolutionary biologists that are equipped with the tools of genomics, speciation theory, and an increasingly sophisticated array of hybrid zone case studies.

An important component in the evolution of the *P. glutinosus* group, and the three focal species especially, is hybridization. This theme emerges, in some form, in every one of the previous chapters. In Chapter II, hybridization is one of the four major problems encountered in delimiting species boundaries in the group. Chapter III deals entirely with hybridization, with emergent patterns clearly indicating differential introgression and possibly hybrid zone movement as well. In Chapter IV, ecological niche modeling is used to place the *P. jordani*-*P. metcalfi* hybrid zone into biogeographic context and give temporal perspective. As technological advances allow for a greater number of molecular markers and increased spatial resolution for ecological niche models, the dynamics presented here may be further elucidated.

With regard to the future of species delimitation in the *P. glutinosus* group, researchers have made, and continue to make, significant progress. Most notably, recent multi-gene phylogenetic analyses are beginning to document widespread paraphyly among complexes that were once thought to be monophyletic (e.g., the *P. glutinosus* and *P. jordani* complexes). Furthermore, these same studies are in disagreement about relationships among species (Palmer et al. 2005, Kozak et al. 2006, Wiens et al. 2006). Besides confirming the genuine difficulty of the group, these studies demonstrate the continued interest in the evolutionary history of the group. As more genes become sequenced and multigene analytical methods refined, relationships among the lineages will almost certainly become clearer.

It is evident that the spatially fine scale analysis presented in Chapter III determined, to a large extent, the ability to infer hybrid zone movement. A logical next step is to increase the number of nuclear DNA markers, so that a more complete picture emerges of the extent of introgression. With enough molecular markers, a hybrid score can be calculated (following Buerkle 2005), and additional patterns of differential introgression may emerge. Of particular interest is red cheek pigmentation, as this trait is under putative positive selection (Huheey 1960, Brodie and Howard 1973, Hensel and

Brodie 1976). Empirical examples of the introgression of adaptively advantageous traits are rare in the literature, and its detection here remains a possibility.

The use of ecological niche modeling has seen a boon in recent years, and has been successfully applied in a few hybrid zone studies (Cicero 2004, Swenson 2006, 2008, Swenson et al. 2008). Its application in this study, however, is novel in the fine-scale at which models are utilized. The work presented here represents a good first step to integrating ecological niche modeling with traditional methods of hybrid zone analyses.

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