5-2019

Organization of genetic and reproductive behavioral diversity in the two-lined salamander (*Eurycea bislineata*) species complex

Todd Warren Pierson
University of Tennessee

To the Graduate Council:

I am submitting herewith a dissertation written by Todd Warren Pierson entitled "Organization of genetic and reproductive behavioral diversity in the two-lined salamander (*Eurycea bislineata*) species complex." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Ecology and Evolutionary Biology.

Benjamin M. Fitzpatrick, Major Professor

We have read this dissertation and recommend its acceptance:

James A. Fordyce, Benjamin P. Keck, Brian O'Meara

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Organization of genetic and reproductive behavioral diversity in the two-lined salamander (*Eurycea bislineata*) species complex

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Todd Warren Pierson

May 2019

For Edward Abbey, whose books taught me the value of sharp wit and reluctant enthusiasm. And for Emmett Reid Dunn, who first captured with words the allure and addiction of plethodontid salamander biology—from the "unalloyed drudgery" to the "peculiar and high satisfaction". *Bueno, vaya usted con Dios.*

Acknowledgments

First, I thank my family and friends for their unwavering support of my academic pursuits. I love you all. In particular, I thank Natalia Bayona-Vásquez for her neverending patience, kindness, and reassurance. I owe great thanks to my primary collaborators on this research—Benjamin Fitzpatrick, Jennifer Deitloff, Carlos Camp, Kenneth Kozak, and Stanley Sessions—for their many contributions. Although I have written this dissertation in the first-person, much of this work was collaborative. More specifically, I conducted Chapter 2 in collaboration with Kenneth Kozak and Benjamin Fitzpatrick, Chapter 3 in collaboration with Carlos Camp and Benjamin Fitzpatrick, and Chapter 4 in collaboration with Jennifer Deitloff, Stanley Sessions, Kenneth Kozak, and Benjamin Fitzpatrick. As presented here, this last chapter is an amalgam of two manuscripts we submitted for publication [141, 140].

I am indebted to my many colleagues, collaborators, and comrades—human and canine—for the hours they've spent alongside me in the field, laboratory, or museum and for the help, advice, and encouragement they've given. These include, but are not limited to, S. Arnold, C. Bayona, N. Bayona-Vásquez, C. Beachy, D. Beamer, J. Beane, S. Bolick, R. Bonett, M. Britz, G. Brown, R. Bruce, C. Carter, E. Carter, J. Clare, D. Cook, J. Corush, J. Costa, J. Cunningham, A. Devault, S. Drukker, A. Edwards, M. Ellison, K. Enge, P. Feldhoff, R. Feldhoff, J. Fetterman, N. Fields, J. Fordyce, A. Funk, C. Gienger, T. Glenn, S. Graham, L. Hayter, B. Holt, B. Keck, T. Kieran, H. Korotkin, J. Lamb, W. Lattea, J. Maerz, Z. Marion, K. McEntire, A. Miele, C. Miller, S. Nelson, M. Nordgren, B. O'Meara, T. Paulson, J. Pechmann, K. Pursel, H. Rainey, G. Righi, T. Ryan, L. Sargent Macauley, N. Scobel, D. Sever, N. Shepard, T. Stratmann, B. Stuart, P. Super, S. Wesnak, J. Wessels, E. White, D. Wilburn, J. Wooten, S. Workman, K. Wray, and K. Young. I am particularly thankful for W. Lattea—the only person I know whose enthusiasm for standing in the rain and watching

salamanders exceeds my own. I thank HerpMapper (www.HerpMapper.org) and its citizen-scientist contributors, as their observations were instrumental in guiding these project.

This research was funded in part by the American Philosophical Society's Lewis and Clark Fund for Exploration and Field Research, the American Museum of Natural History's Theodore Roosevelt Memorial Grant, the Society of Systematic Biologists's Graduate Student Research Award, University of Tennessee Knoxville's Department of Ecology and Evolutionary Biology's Departmental Research Funds, Lock Haven University's Biological Sciences Department and Faculty Professional Development Committee, the North Carolina Herpetological Society, and the Highlands Biological Station's Grant-in-Aid (Bruce Family Scholarship in Herpetology). Throughout my time at the University of Tennessee Knoxville, I was supported by a National Science Foundation Graduate Research Fellowship (DGE-1452154).

This research was approved under animal use protocols from the University of Tennessee Knoxville IACUC (2621-0618, 2482-0916, 2352-0515, 2471-0616), Lock Haven University IACUC (01501), Hartwick College IACUC (SKS-11), and Highlands Biological Station IACUC (HBS-18-06). This research was permitted under an Alabama Department of Conservation and Natural Resources Scientific Collection Permit, North Carolina Wildlife Resources Commission Wildlife Collection Licenses (15-SC00977, 18-SC01274) and an Endangered Species Permit (15-ES00441), Tennessee Wildlife Resource Agency Scientific Collection Permits (3840, 1213), Virginia Department of Game and Inland Fisheries Scientific Collection Permits (053323, 058754), a Tennessee State Forest Research Permit (Allforests-2015-01), a Tennessee Department of Environment and Conservation Scientific Research and Collection Permit (2015-006), permission from the Bankhead, Chattahoochee, Nantahala, Pisgah, Cherokee, George Washington and Jefferson National Forests, and Scientific Research and Collecting Permits from Great Smoky Mountains National Park (GRSM-01198) and the Obed Wild and Scenic River (OBRI-00045).

Abstract

Describing the mechanisms that generate and maintain genetic and phenotypic diversity is a central goal of evolutionary biology. This is dependent upon accurate reconstructions of evolutionary histories, which have traditionally been inferred as simple, bifurcating phylogenies. However, speciation is often neither instantaneous nor permanent, and genomic data have revealed the ubiquity of reticulate evolutionary histories across diverse organisms. This revelation has underscored the importance of understanding the causes and outcomes of ancient and modern hybridization. Here, I examine these themes by studying the distribution of genetic and reproductive behavioral diversity in the two-lined salamander (*Eurycea bislineata*) species complex—a group of plethodontid salamanders. First, I use a phylogenomic methods with dataset of $>$ 2 million SNPs from $>$ 120 individuals to demonstrate the importance of river drainage reorganization in the reticulate evolutionary history of the group. Next, I use population genomic methods and a dataset of $>$ 9,000 SNPs from 330 individuals in replicate contact zones to demonstrate reproductive isolation and fine-scale ecological segregation between two sympatric species. Finally, I use a genomic, behavioral, and field observational data to demonstrate the existence of alternative reproductive tactics within three putative species in the group and comment more broadly on variation in reproductive behavior. Together, these results demonstrate previously unrecognized genetic and behavioral variation in the *E. bislineata* species complex and describe the mechanisms—from large-scale geologic change to fine-scale ecological gradients—responsible for its organization.

Table of Contents

1 Introduction **1**

1.1 Ancient hybridizaion and reticulate evolution 2

1.2 Secondary contact and ecological divergence 3

1.3 Inter- and intraspecific variation of reproductive morphology and behavior . 5

1.3.1 Alternative reproductive tactics 6

1.4 The *Eurycea bislineata* species complex 7

2 River drainage reorganization, hybridization, and phylogeography **10**

2.1 Introduction 10

2.1.1 Unresolved phylogenetic discordance 11

2.1.2 Fragmentation of White's River 12

2.1.3 Formation of the modern Tennessee River 13

2.1.4 Objectives 14

2.2 Methods 15

2.2.1 3RAD library preparation and sequencing 15

2.2.2 3RAD data assembly 16

2.2.3 Phylogenetic analyses 16

2.2.4 Principal components analyses 17

2.2.5 D-statistics 18

2.2.6 Migration models using coalescent simulations 18

2.3 Results 19

2.3.1 Phylogenetic analyses 19

2.3.2 Principal components analyses 21

2.3.3 D-statistics . . . 22
2.3.4 Migration models using coalescent simulations . . . 22
2.4 Discussion . . . 22
2.4.1 Evolutionary history of the *E. bislineata* species complex . . . 22
2.4.2 Reorganization of White's River . . . 24
2.4.3 Formation of the modern Tennessee River . . . 26
2.4.4 Additional secondary contact and introgression . . . 27
2.4.5 Summary . . . 28

3 Fine-scale ecological segregation in replicate contact zones 31
3.1 Introduction . . . 31
3.1.1 Contact zones between northern and southern clades . . . 32
3.1.2 Contact zones within northern or southern clades . . . 32
3.1.3 Objectives . . . 34
3.2 Methods . . . 35
3.2.1 Sampling and environmental data collection . . . 35
3.2.2 RADcap bait design . . . 35
3.2.3 RADcap library preparation and sequencing . . . 36
3.2.4 RADcap data assembly . . . 38
3.2.5 Population assignment using `Structure` and DAPC . . . 38
3.2.6 Comparison of habitat characteristics . . . 39
3.2.7 Comparison of species composition among stream habitats . . . 40
3.3 Results . . . 40
3.3.1 Population assignment using `Structure` and DAPC . . . 40
3.3.2 Comparison of habitat characteristics . . . 41
3.3.3 Comparison of species composition among stream habitats . . . 41
3.4 Discussion . . . 41

4 Alternative reproductive tactics 44
4.1 Introduction . . . 44
4.1.1 Objectives . . . 46

4.2 Methods . . . 47
4.2.1 Phylogenetic analysis . . . 47
4.2.2 Behavioral trials . . . 48
4.2.3 Karyological analysis . . . 48
4.2.4 Preliminary field observations . . . 49
4.2.5 Additional field observations . . . 49
4.3 Results . . . 51
4.3.1 Phylogenetic analysis . . . 51
4.3.2 Behavioral trials . . . 51
4.3.3 Karyological analysis . . . 52
4.3.4 Preliminary field observations . . . 52
4.3.5 Additional field observations . . . 52
4.4 Discussion . . . 53

5 Conclusion **59**

Bibliography **60**

Appendix **82**
A Tables . . . 83
B Figures . . . 91
C Supplemental Tables . . . 109
D Supplemental Figures . . . 140

Vita **146**

List of Tables

Table 1 Results of Shimodaira–Hasegawa tests 83
Table 2 Results of D-statistics 84
Table 3 Migration model comparison using `fastsimcoal2` 85
Table 4 Karyotypes for select *Eurycea* cf. *wilderae* 86
Table 5 Results of preliminary field surveys for *Eurycea* cf. *wilderae* 87
Table 6 Observations of *Eurycea* cf. *wilderae* from funnel traps 88
Table 7 Observations of *Eurycea* cf. *wilderae* from nocturnal forest floor surveys 89
Table 8 Observations of *Eurycea* cf. *wilderae* from stream surveys 90
Table S1 Phylogeography sampling localities 109
Table S2 `Structure` results 114
Table S3 DAPC results 127

List of Figures

Figure 1 Named taxa in *Eurycea bislineata* species complex 91
Figure 2 Maps of paleodrainage and modern river drainage basins 92
Figure 3 Phylogeography sampling localities. 93
Figure 4 Maximum-likelihood phylogenies from *full_min5*, *north*, and *south* . . 94
Figure 5 Phylogenetic networks 95
Figure 6 Principal components analyses 96
Figure 7 D-statistics 97
Figure 8 `fastsimcoal2` models 98
Figure 9 Map of replicate contact zones. 99
Figure 10 Stream habitat vs. species assignment 100
Figure 11 Stream habitat characteristics 101
Figure 12 Alternative reproductive tactics in *Eurycea* cf. *wilderae* 102
Figure 13 Sampling map for alternative reproductive tactics 103
Figure 14 Maximum-likelihood phylogeny of reproductive tactics in *Eurycea* . . 104
Figure 15 *Eurycea* behavioral trial data 105
Figure 16 Chromosome spread from *E.* cf. *wilderae* 106
Figure 17 Spatial distribution of breeding *Eurycea* cf. *wilderae* 107
Figure 18 Snout-vent length:head width ratios 108
Figure S1 Phylogeography sampling localities. 140
Figure S2 Maximum-likelihood phylogeny from *full_min30* 141
Figure S3 Phylogenetic networks with sample labels 142
Figure S4 Principal components analyses with sample labels 143
Figure S5 Distribution of locus-specific F_{ST} 144

Figure S6 Linear distribution of stream habitats 145

Chapter 1

Introduction

Describing the mechanisms that generate and maintain genetic and phenotypic diversity is a central goal of evolutionary biology. This is dependent upon accurate reconstructions of evolutionary histories. Early methods designed for this task were hindered by morphological conservatism and homoplasy—challenges that were greatly ameliorated with the introduction and development of DNA sequencing and molecular phylogenetic methods. However, most of these traditional methods implicitly assume that speciation is instantaneous and permanent, leading to bifurcating phylogenies. The advent of genome-scale data and accompanying phylogenomic methods have revealed the ubiquity of reticulate evolutionary histories and the importance of introgressive hybridization in a wide diversity of organisms [179], including humans [62]. This revelation has underscored the importance of research investigating the geological and climatic processes creating the opportunity for secondary contact [76, 177], the patterns of gene flow at contemporary contact zones [9, 185], and the phenotypic and behavioral diversity structuring that gene flow [90, 45].

The lungless salamanders of the family Plethodontidae are particularly compelling models for studying these themes [192]. The $>$ 470 species in this clade last shared a common ancestor in the Cretaceous [166] and include fully aquatic, semiaquatic, and fully terrestrial forms [138, 4]. Because they have low dispersal abilities, most species show strong phylogeographic structure, and the family includes many groups of morphologically similar species with allopatric or parapatric distributions [192, 4, 77, 95]. For these reasons, lessons learned from plethodontid salamanders have often played outsized roles in the fields of

phylogeography and systematics [129, 95, 106, 191, 148]. The old age of this group and its strong phylogeographic patterns suggest that it may also prove useful for understanding the role of ancient and modern gene flow in structuring the current distribution of genetic diversity. Below, using examples from plethodontid salamanders, I discuss the general themes of 1) ancient hybridization and reticulate evolution; 2) secondary contact and ecological divergence; and 3) inter- and intraspecific variation in reproductive morphology and behavior.

1.1 Ancient hybridizaion and reticulate evolution

Most speciation occurs in allopatry [123, 34], and phylogeographic studies of sessile organisms often reveal a close relationship between biogeographic barriers and phylogeny [8]. For example, many terrestrial plethodontid salamanders have allopatric or parapatric species boundaries that largely coincide with major rivers [78, 77] or regions with inhospitable climates [78, 95]. For aquatic organisms living in lotic systems, the organization of river drainages is strongly predictive of phylogeny, and river drainage divides operate as important biogeographic barriers—a phenomenon that is particularly well described in fish [82]. Few plethodontid salamanders are fully aquatic, but many species have biphasic life histories, with aquatic egg deposition, aquatic larvae, and semiaquatic adults [138]. Phylogeographic studies of aquatic and semiaquatic plethodontid salamanders have indeed demonstrated a correspondence between river drainage boundaries and phylogeny [99, 106, 111, 38], underscoring the importance of drainage divides in creating vicariance and promoting diversification in this group.

Secondary contact occurs when two lineages, previously evolving independently in allopatry, are brought back into sympatry. It can be initiated by geologic or climatic change that removes or diminishes biogeographic barriers (e.g., the reduction in size of a river) [76]. Dependent upon factors such as the degree of reproductive isolation and ecological niche divergence between these lineages, secondary contact can yield outcomes from widespread sympatry to unencumbered hybridization. Genome-scale data from diverse organisms not currently in sympatry have revealed signatures of ancient hybridization, implying the existence of previous periods of secondary contact [23, 179]. In some plethodontid

salamanders, discordance between various molecular and morphological datasets has been interpreted as evidence of ancient hybridization and introgression, potentially driven by geological or climatic change that altered biogeographic barriers [95, 196].

For aquatic organisms living in lotic systems, secondary contact may be initiated by river drainage reorganization. Gradual changes—like divide migration—are driven by processes such as differential erosion and sediment deposition over millions of years. Other changes may be driven by climate, with proximate causes including glaciation and sea-level change. More discrete, instantaneous changes include stream capture—in which the headwaters of one river erode upstream, intersect another river channel, and divert its waters [14]. These various mechanisms of river drainage reorganization have long been used to explain the biogeography of aquatic taxa [82, 122, 24], and because the Appalachian highlands of the eastern United States are home to a tremendous diversity of freshwater fish, crayfish, mussels, and salamanders, this region has been the focus of many such studies. Geological and biological data support a dynamic hydrological history in the region, with most research focused on changes occurring from the Miocene to the present [82, 137]. In Appalachia and elsewhere, river drainage reorganization has primarily been invoked as an explanation for the vicariance and dispersal of aquatic taxa, but in the absence of reproductive isolation, these changes may also initiate gene flow between previously independently evolving taxa. Comparatively few studies—largely due to limitations imposed by the number and diversity of molecular markers—have focused on the role of river drainage reorganization in the initiation of secondary contact, hybridization, and introgression.

1.2 Secondary contact and ecological divergence

Because speciation occurs primarily in allopatry, secondary contact provides a unique opportunity to investigate hybridization, reproductive isolation, and niche divergence. Thus, these contact zones have long been considered a "natural laboratory for evolutionary studies" [75] and a "window on the evolutionary process" [69], revealing mechanisms otherwise difficult to observe in nature.

In the absence of reproductive isolation, secondary contact initiates hybridization. In their simplest form, stable hybrid zones (i.e., tension zones) exist as a balance between dispersal and selection against hybrids [66, 9]. When selection is dependent upon environment, a strong association between genotype and environmental features can manifest in a cline, where each parental genotype dominates in one habitat and hybridization occurs at the ecotone [66]. In perhaps the most famous plethodontid salamander—the "ring species" *Ensatina eschscholtzii*—some hybrid zones are very narrow, occur at ecotones, and are maintained by strong selection against hybrids [193, 2, 37]. In contrast, among some members of the genus *Plethodon*, hybrid zones situated along elevational and ecological gradients appear to be quite wide, and most individuals in these "hybrid swarms" are morphologically intermediate [194, 78]. In other organisms, the patchy distribution of ecotones and opportunities for hybridization can create mosaic hybrid zones [68, 71, 146]. The outcome of hybridization can be further structured by heterogeneity among individuals, such as when mate-choice causes asymmetric introgression of sexual traits [204], and by heterogeneity within genomes, as independent assortment and recombination allow alleles to segregate from their parental genomes and introgress at variable rates [9, 21]. An emphasis on this intragenomic variation in introgression has encouraged the view of genomes as semipermeable entities, and subsequent effort has focused on identifying "genomic islands"—the regions of the genome that resist introgression and maintain species identities [70].

When species boundaries are maintained—either through persistent genomic islands or the complete reproductive isolation—contact zones also prove informative about ecological divergence and the mechanisms of community assembly, including competition. The competitive exclusion principle predicts the instability of sympatry between species with identical ecological niches [67]. If two species are observed in sympatry and have divergent ecological niches, these differences can be explained by either: 1) sorting of preexisting niche divergence [25]; or 2) novel divergence driven by competition (e.g., character displacement) [16, 39]. Preexisting niche divergence may be uncommon in species complexes of closely related plethodontid salamanders. Macroevolutionary studies have suggested that niche conservatism—in which organism's ecological niche is retained through evolutionary time [201]—was an important driver of diversification for some North American plethodontid

salamanders, including the speciose genera *Plethodon* and *Desmognathus* [108, 109; but see 206]. Many inferences about ecological niche divergence are made from observations of species in allopatry, but contact zones provide one means for testing ecological niche divergence directly and *in situ*. For example, Jaeger [92] experimentally demonstrated that competition from *Plethodon cinereus* excludes the sympatric *P. shenandoah* from forest floor habitats. Similarly, Camp et al. [28] showed that two members of the *Desmognathus quadramaculatus* species complex have similar ecological niches (i.e., preference for small or large streams) in allopatry, but diverge in sympatry—an example consistent with niche compression caused by competition [119]. Thus, contact zones provide a unique opportunities to study the ecological factors responsible for the spatial distribution of genes and species.

1.3 Inter- and intraspecific variation of reproductive morphology and behavior

In some cases, reproductive isolation is predicted by phenotypic trait variation. This can result from simple mechanical incompatibilities between individuals (e.g., the "lock-and-key" model) [42, 170] or perhaps more often, by sexual selection (and reinforcement) disfavoring heterospecifics [33]. Jockusch and Wake [95] describe one example in plethodontid salamanders, noting that in *Batrachoseps*, most examples of gene flow between distantly related species occur between species with similar gross morphology. While general morphology may predict reproductive isolation, differences in reproductive morphology and behavior might be especially influential [124].

Plethodontid salamanders perform ritualized courtship prior to internal fertilization, a process that has been extensively detailed in comparative studies [7, 87]. This often involves tactile stimulation and the delivery of courtship pheromones from males to females, culminating in a behavior called "tail straddle walk" that immediately precedes the external transfer of a spermatophore and subsequent insemination. Most morphological features and behaviors involved in this courtship are remarkably conserved through tens of millions of years of evolution [85]. Variation that does exist (e.g., in the method of pheromone

delivery) is still typically strongly conserved within smaller evolutionary lineages, and the tendency for stereotypy of morphological and behavioral reproductive traits suggests the potential for variation in these traits to reflect species boundaries. Experimental studies have demonstrated strong premating reproductive isolation in some plethodontid salamanders, a mechanism that appears to be important for maintaining some species boundaries [86, 187, 188, 190]. Heterospecific courtship is initiated less often and fails more often in heterospecific pairs than conspecific pairs, and these differences have been attributed both to variation in courtship behavior and pheromone composition [189]. While some studies have described variation in courtship success within species and populations [84, 12], there are not yet any documented examples of dramatic intraspecific differences in reproductive morphology and correlated behavior, such as the alternative reproductive tactics found in other organisms.

1.3.1 Alternative reproductive tactics

Alternative reproductive tactics (ARTs) are discrete behaviors affecting how or when reproduction occurs and are sometimes accompanied by marked morphological differences. ARTs are present in many group of animals [63, 134], and the coexistence of such alternative tactics within populations has been considered particularly enigmatic [e.g., 50].

Much theoretical work has focused on the scenario in which sexual selection leads to high reproductive investment by "bourgeois" individuals, enabling "parasitic" same-sex competitors to exploit these investments [178]. For example, bourgeois male sunfish build and guard nests, while parasitic "sneaker" males stealthily release sperm into other males' nests [64, 139]. In some frogs, parasitic "satellite" males intercept females that bourgeois males have attracted by calling [198]. Many of these polymorphisms are maintained by frequency-dependent selection, with the parasitic tactic unable to exist in isolation.

However, a second scenario might also be common. Here, balancing selection arises from discontinuous environmental or temporal heterogeneity in which tactic has higher fitness. For example, some insects have winged males that are more effective at locating low-density females among patches of suitable habitat [114] and wingless males that are sessile, but have greater fecundity per mating [115], and this polymorphism is maintained

by habitat diversity. Some amphipods have dominant "major" males and smaller "minor" males, and this polymorphism is likely maintained in part by natural selection imposed by seasonally variable food resources [113]. These polymorphisms are characterized by divergent reproductive niches rather than a bourgeois–parasitic relationship.

Under this second scenario, theory predicts that the fixation of one tactic in a population or species (i.e., an evolutionarily stable strategy) or the maintenance of multiple tactics in a stable polymorphism should depend upon local conditions, much like any niche polymorphism [e.g., 22, 169, 74]. Accordingly, ARTs are evolutionarily labile, and repeated gains and losses of similar strategies are common among animals [3]. However, there are few examples of clades including some species fixed for one tactic, some fixed for the other, and some polymorphic species expressing both alternatives.

Zamudio and Chan [209] review ARTs in amphibians and note that in contrast to those of many other vertebrates, none are known to be heritable [but see 174]. Most amphibian ARTs are rather ephemeral and conditional, with individuals capable of switching between alternative tactics on time scales ranging from minutes to seasons. Few are accompanied by marked morphological differences. Facultative paedomorphosis may qualify as a counterexample, but it is often considered a habitat-use polymorphism rather than an outcome of sexual selection [202, 209]. Common forms of ARTs in amphibians include caller vs. satellite males in anurans [198] and female mimicry in salamanders—including in plethodontids [7]. Both of these examples may fall within the broader bourgeois–parasitic category of ARTs [178]. In salamanders, documented ARTs related to resource defense appear to be limited to the few groups with external fertilization (e.g., Cryptobranchidae and Hynobiidae) [103, 186] and are not documented in plethodontids.

1.4 The *Eurycea bislineata* species complex

Here, I use the two-lined salamander (*Eurycea bislineata*) species complex to investigate reticulate evolutionary processes, reproductive isolation and ecological divergence in zones of secondary contact, and the distribution of variation in reproductive morphology and behavior. This is a group of semiaquatic plethodontid salamanders widely distributed

east of the Mississippi River in the United States and Canada [Figure 1; throughout this dissertation, all tables are available in Appendix A, all figures are available in Appendix B, all supplemental tables are available in Appendix C, and all supplemental figures are available in Appendix D].

Eurycea bislineata was described by Green [60], and subsequent investigations of morphological variation within this species resulted in the recognition of four subspecies by Mittleman [128]: 1) *E. b. bislineata* in the northeastern United States [60], which included several synonymized forms [135, 44, 128, 163]; 2) *E. b. cirrigera* in the Gulf and Atlantic Coastal Plains [61]; 3) *E. b. wilderae* in the southern Appalachian Mountains [43] ; and 4) *E. b. rivicola* in much of the Ohio River Valley [127]. Also based on morphological characters, Rose and Bush [150] described *Eurycea aquatica* as a distinct species from Alabama, and Sever et al. [164] described *Eurycea junaluska* from western North Carolina. Later authors questioned the validity of *E. aquatica* [130, 100, 162], and Sever [160] recommended the placement of *E. b. rivicola* in synonymy with *E. b. cirrigera* due to a lack of morphological distinctiveness.

The advent of molecular genetic data provided more resolution. Jacobs [91] used allozyme data to provide the first thorough assessment of molecular genetic variation in the group. He recommended, through the elevation of existing subspecies, the recognition of *E. cirrigera* and *E. wilderae* as distinct species, leaving some ambiguity as to how *E. aquatica* and *E. junaluska* should be treated. Kozak et al. [106] used the phylogenetic analysis of mitochondrial DNA sequence data to reevaluate the group. This study demonstrated the paraphyly of *E. cirrigera* and *E. wilderae* and the distinctiveness of *E. aquatica* and *E. junaluska*, and although it suggested much more diversity than was reflected by the available taxonomy, it did not make any new taxonomic recommendations. Finally, Timpe et al. [182] used a phylogenetic approach to convincingly demonstrate the distinctiveness of *E. aquatica* from sympatric *E. cirrigera*. Thus, most taxonomic authorities currently recognize five species: *E. aquatica, E. bislineata, E. cirrigera, E. junaluska*, and *E. wilderae* [4].

A strong correspondence between paleo- and modern river drainage divides and putative species boundaries in the *E. bislineata* species complex and discordance between allozyme [91] and mitochondrial [106] datasets make this group a compelling model for studying reticulate

evolutionary processes. Because at least one region of secondary contact is well-delineated, this group is also useful for studying hybridization and ecological divergence. Finally, because substantial intra- and interspecific variation exists in reproductive morphological and behavioral traits in the *E. bislineata* species complex [160, 147], it is useful for studying the evolution and implications of this diversity.

Chapter 2

River drainage reorganization, hybridization, and phylogeography

2.1 Introduction

Because their alternative means of dispersal among river drainages are limited, fully aquatic organisms (e.g., fish and mussels) are the most suitable models for studying the influence of river drainage reorganization on the distribution of genetic diversity. In contrast, the semiaquatic *Eurycea bislineata* species complex is capable of overland movements, providing an *a priori* expectation for weaker correspondence between its evolutionary history and the organization of river drainages. In at least some populations, adults make upslope, overland migrations following reproduction in streams [120, 138]. However, these migrations are limited in distance (mean = 65 m) [120], and the strength of breeding-site philopatry is unknown. In contrast, field studies have demonstrated frequent, downstream-biased movement by aquatic larvae [97, 175, 18]. Within populations, this generates a positive correlation between stream slope and gene flow [118]. Thus, gene flow in the *E. bislineata* species complex may occur primarily within, and not among, two-dimensional stream corridors, much likely fully aquatic organisms. Furthermore, downstream-biased movements predict that headwater stream captures may have a rapid and far-reaching influence on the distribution of genetic diversity.

It is unsurprising, then, that the *E. bislineata* species complex consists of species with primarily parapatric distributions and boundaries coincident with river drainage divides [106]. This group last shared a common ancestor in the Miocene (6–22 mya) [91, 106, 15, 207], and most divergences between pairs of putative sister species occurred from the mid-Miocene to late-Pliocene [106]. Consistent with these dates, Kozak et al. [106] demonstrated that the mitochondrial (mtDNA) phylogeny of the *E. bislineata* species complex is better explained by the hypothesized geography of Miocene and Pliocene paleodrainages—including the Appalachian River, Old Ohio, Cumberland, and Tennessee Rivers, the Teays River, and the Laurentian River—than by modern river drainages [Figure 2]. This study suggested that subsequent reorganization of these paleodrainages promoted further vicariant speciation, dispersal, and secondary contact.

2.1.1 Unresolved phylogenetic discordance

Although five species are currently recognized (*E. bislineata, E. aquatica, E. junaluska, E. wilderae, and E. cirrigera*) [Figure 1], Kozak et al. [106] demonstrated the paraphyly of three of these taxa. His study recognized two major clades—"northern" and "southern"—and informally named major mtDNA groups within each as "lineages" (e.g., "Lineage M"). Because the utility of paraphyletic names is limited, in the remainder of this dissertation, I will frequently use these lineage names to describe putative species within the group. Many of the relationships among the lineages inferred by Kozak et al. [106] are concordant with allozyme [91] and morphological [162] characters, but two notable exceptions include: 1) the monophyly of *E. wilderae* (Lineages E, J, and M); and 2) the phylogenetic position of *E. junaluska* (Lineage G) and *E. aquatica* (Lineage H).

Eurycea wilderae is a gracile, brightly-colored form found primarily at high elevations and thus is morphologically and ecologically distinct from other members of the *E. bislineata* species complex [158, 162]. Using allozyme data, Jacobs [91] recovered two distinct groups within this putative species—one from southwestern Virginia and neighboring North Carolina and Tennessee and one extending southward throughout the remainder of the southern Appalachian Mountains. Although he did not resolve the monophyly of these groups, Jacobs [91] recommended the elevation of *E. wilderae* from subspecific status. Later, Kozak

et al. [106] recovered three paraphyletic mtDNA lineages—Lineages E, J, and M—within *E. wilderae*, with the former belonging to the northern clade and the latter two to the southern clade. Thus, as it stands, there is strong evidence that this morphologically distinct taxon is actually composed of multiple cryptic species or reflects admixture between northern and southern clades.

Eurycea aquatica is a larger, more robust form found primarily in springs in the Ridge and Valley physiographic region [150]. *Eurycea junaluska* is likewise a relatively large species, but it is more brightly colored and often lacks distinct dorsolateral stripes; it is found in only three small subdrainages of the Tennessee River in the southern Appalachians [138]. Jacobs [91] recovered a sister relationship between *E. aquatica* and *E. junaluska* and suggested the close affinity of both to *E. cirrigera* from the Ohio River Valley, with which they also share morphological similarities. These latter populations include Lineages C and D and belong to the northern clade, as described by Kozak et al. [106]. Kozak et al. [106] likewise recovered a sister relationship between Lineages G and H, but instead inferred their membership to southern clade, which includes geographically proximate but morphologically divergent lineages. Thus, while the sister relationship between these two taxa is well-supported, a consensus is lacking regarding their broader position within the *E. bislineata* species complex.

These two examples of discordance between morphological, allozyme, and mitochondrial datasets may simply reflect incomplete lineage sorting, homoplasy, or the limits of the data available for these studies. Alternatively, they may be suggestive of a more complex, reticulate evolutionary history in this group. The latter hypothesis is supported by the importance of river drainage reorganization in the evolutionary history of the *E. bislineata* species complex and the geographic proximity of each example to hypotheses of contemporaneous and large-scale paleodrainage reorganization: the fragmentation of White's River and the formation of the modern Tennessee River, respectively [Figure 2].

2.1.2 Fragmentation of White's River

Inspired by geological data and anomalies in modern drainage patterns, White [200] hypothesized a Pliocene paleodrainage with headwaters reaching just east of Asheville, North Carolina and draining northeast through Virginia. Until it was reorganized into modern

Atlantic Slope drainages, this putative "White's River" [208] primarily encompassed the headwaters of the modern Catawba and Yadkin–Peedee River drainages and may have had an outlet through the modern Roanoke River [Figure 2]. The former existence of White's River is consistent with some biogeographic patterns in the region and has been invoked to explain the distributions of local fish [205, 131, 152], crayfish [32], mussels [155], and salamanders [72]. However, this hypothesis remains controversial among both geologists and biologists. For example, Dietrich [40] argued that White's River likely never existed, while Wood and Mayden [205] suggested its headwaters extended even further than originally hypothesized, approaching the modern Chattahoochee River in Georgia. If White's River existed, it likely would have been bordered to the north by the Teays River paleodrainage, about which there is a greater consensus [Figure 2]. Before being rerouted during the last glacial period, the Teays River flowed north through the route of the modern New–Kanawha River, turned west, and eventually joined the Mississippi River separate from the Old Ohio, Cumberland, and Tennessee Rivers [93]. The headwaters of the Teays River remain largely intact as the modern New River, but multiple smaller stream capture events have been proposed between it and neighboring drainages, including those from the former White's River [81, 80].

The former course of the hypothesized White's River primarily encompassed the modern distributions of Lineages E, I, and J, and the Teays River paleodrainage encompassed the modern distribution of Lineage E [106]. Thus, the region is a contact zone between the northern and southern clades, including one lineage currently assigned to the paraphyletic *E. wilderae* from each clade. This suggests that the history of river drainage reorganization might be relevant to biogeography of this taxon. Indeed, although he did not explicitly discuss White's River, Kozak et al. [106] hypothesized that Lineage J expanded its distribution from the Upper Catawba River drainage to the Atlantic Coastal Plan, perhaps consistent with the formation of its rivers following the fragmentation of White's River.

2.1.3 Formation of the modern Tennessee River

The formation of the modern Tennessee River is particularly enigmatic, and its unusual drainage pattern and abrupt changes of direction have long fueled speculation that it is a

composite of multiple paleodrainages. In what has become perhaps the most influential explanation, Hayes and Campbell [73] hypothesized the existence of an "Appalachian River" following the course of the modern upper Tennessee River and draining through the modern Coosa River into the Gulf of Mexico. Then, through a series of sequential captures, the modern upper Tennessee River was rerouted to its present course in the late Miocene [54]. The Appalachian River hypothesis has great appeal to biologists, as it provides an explanation for shared biota among the Tennessee and Mobile drainages [183, 151, 51]. However, numerous criticisms of, modifications of, and replacements for this hypothesis—founded upon both geological and biological evidence [e.g., 199, 96, 151, 31]—have prevented a consensus regarding precise history of drainage reorganization [137]. Additional hypothesized drainage reorganization events in the region further complicate its hydrological history, but reinforce the consensus that it has been dynamic [151, 145]. The former course of the upper Appalachian River is now occupied by (among others) Lineages G and H, and Kozak et al. [106] suggested that the divergence between these two species may have followed the formation of the modern Tennessee River. If drainage reorganization also initiated introgressive hybridization with other members of the *E. bislineata* species complex, it may help explain the difficulty in resolving the phylogenetic position of these lineages.

2.1.4 Objectives

Here, I reevaluated the evolutionary history of the *E. bislineata* species complex using genome-scale data, focusing on determining whether ancient introgressive hybridization is responsible for incongruities identified in previous studies that used morphological, allozyme, and mtDNA datasets. Failure to account for introgressed alleles can bias phylogenetic inferences and generate misleading conclusions about evolutionary histories [116, 48], so I used a suite of methods—including the comparison of phylogenetic inferences with "full" and "censored" data matrices, phylogenetic networks, principal components analyses, D-statistics, and migration models using coalescent simulations—to evaluate evidence for a reticulate evolutionary history in this group.

Because the early evolutionary history of the *E. bislineata* species complex was influenced by the organization of paleodrainages [106], it stands to reason that ancient introgressive

hybridization would be centered in regions of large-scale drainage reorganization, such as those surrounding the hypothesized White's River and Appalachian Rivers. The existence, exact course, and history of these paleodrainages are the subjects of great debate among geologists and biologists, and resolving these questions is beyond the scope and ability of this study. However, while disagreeing in detail, most competing hypotheses agree upon the general dynamism of river drainage reorganization in these two regions, suggesting that they are likely centers of secondary contact between previously independently evolving lineages. Indeed, both are within or near the greatest "phylogeographic break hotspots" for amphibians in North America [149], and both include contact between northern and southern clades of the *E. bislineata* species complex [106, 91]. If discordance among previous datasets is evidence of a reticulate evolutionary history of this group, these two regions may be particularly important for understanding the predictors and outcomes of ancient hybridization. Thus, where appropriate, I interpret results with reference to these paleodrainage hypotheses.

2.2 Methods

2.2.1 3RAD library preparation and sequencing

I created 3RAD libraries [11] for 119 samples of the *Eurycea bislineata* species complex, representing all major mitochondrial lineages described in Kozak et al. [106], plus the enigmatic "Sandhills *Eurycea*" [91] not included in that study and hereafter referred to as "Lineage S" [Figure 3; Table S1]. I also created libraries for two samples of *Eurycea [Haideotriton] wallacei*, which some previous studies had recovered as nested within this group, and three outgroup samples representing *Eurycea guttolineata*, *Eurycea longicauda*, and *Eurycea quadridigitata*. I primarily used samples from Kozak et al. [106], and I extracted DNA from additional fresh tissue samples using Qiagen DNeasy Blood and Tissue Kits. I prepared libraries using the enzymes XbaI, NheI-HF, and EcoRI and conducted individual PCRs following Bayona-Vásquez et al. [11]. I quantified final libraries using a Qubit 2.0 fluorometer and pooled samples accordingly. I size-selected most libraries for 500 bp +/-

10% on a Pippin Prep; for some additional samples that I prepared at a later date (E118–E124), I adjusted for expected inconsistencies in the size-selection process by selecting for a broader range (i.e., 500 +/- 15%). I cleaned these products using SpeedBeads, quantified them again using a Qubit 2.0 fluorometer, and pooled them with libraries from unrelated projects for sequencing Illumina NextSeq PE75 or Illumina HiSeq PE150 runs.

2.2.2 3RAD data assembly

I demultiplexed all reads by external indexes (i.e., iTru7/iTru5) [Table S1] using `bcl2fastq` (Illumina, Inc.). I removed internal indexes, quality-filtered, and trimmed reads to 62 bases using step1 of `ipyrad` v0.7.19 [47, 46] separately for each sample. I then assembled these data *de novo* using step1—step7 in `ipyrad`, using a clustering threshold of 0.85, a minimum depth for statistical base calling of 6, and a minimum depth for majority-rule base calling of 3. I exported four assemblies: 1) *full_min5*, which included all samples and only loci present in a minimum of five in-group samples; 2) *full_min30*, which included all samples and only loci present in a minimum of 30 in-group samples; 3) *north*, which included only samples from the northern clade [as redefined in Results; Figure 4] and only loci present in a minimum of five in-group samples; 4) *south*, which included only samples from the southern clade [as redefined in Results; Figure 4] and only loci present in a minimum of five in-group samples. I assembled all data using computing resources from the Advanced Computing Facility (ACF) that I accessed through the University of Tennessee Knoxville.

2.2.3 Phylogenetic analyses

For each assembly, I estimated a maximum-likelihood (ML) phylogeny in `RAxML-NG` [110] using a concatenated matrix of one SNP per locus (i.e., the .u.snps.phy output from `ipyrad`) and a GTR+G+ASC_LEWIS substitution model. I rooted each phylogeny with *E. quadridigitata* (E114), and I assessed confidence using 100 bootstrap replicates. I conducted all inferences on an Amazon EC2 Instance.

One way to identify samples with introgressed alleles is through the comparison of "full" and "censored" datasets, similar to Eaton and Ree [48]. In brief, when reconstructing

a phylogeny, alleles shared due to reticulations will be interpreted as if they were alleles shared due to common ancestry, sometimes altering inferred topologies. By removing the "donor" of these alleles and reinferring the phylogeny from this censored dataset, the effect of introgression on the topology can be assessed. I conducted three such tests: 1) using the *north* assembly, I compared the ML topology inferred from the *north* assembly vs. the alternative ML topology inferred from the *full_min5* assembly, pruned to include only taxa present in *north*; 2) using the *south* assembly, I compared the ML topology inferred from the *south* assembly vs. the alternative ML topology inferred from the *full_min5* assembly, pruned to include only taxa present in *south*; 3) using the *full_min5* assembly, I compared the ML topology inferred from the *full_min5* assembly vs. an alternative, composite topology created from ML topologies inferred from the *north* and *south* assemblies. I compared likelihoods of ML vs. alternative topologies using Shimodaira–Hasegawa (SH) tests ($\alpha = 0.05$) [167] as implemented in `RAxML` v8.2.11 [171]. For each test, I used the respective concatenated matrix of one SNP per locus (i.e., the .u.snps.phy output from `ipyrad`) and a GTRGAMMA substitution model.

Finally, to visualize preliminary evidence of reticulate evolutionary histories, I constructed distance-based phylogenetic networks using the Neighbor-Net algorithm [20] implemented in the package `phangorn` [154, 153] in `R` v3.5.1 [144]. I constructed three networks, each using Hamming distance matrices calculated from one random SNP per locus (i.e., the .u.snps.phy output from `ipyrad`) from the *full_min30* assembly: 1) all samples, excluding outgroups and *E. wallacei*; 2) only samples in the northern clade; and 3) only samples in the southern clade.

2.2.4 Principal components analyses

To provide an alternative perspective on the distribution of genetic diversity in the *E. bislineata* species complex not dependent upon a bifurcating phylogeny, I conducted principal components analyses (PCA) using the package `ade4` [41] in `R` v3.5.1 [144]. I conducted three PCAs, each using the matrix of one random SNP per locus (i.e., the .ustr output file from `ipyrad`) from the *full_min5* assembly: 1) all samples, excluding outgroups and *E. wallacei*;

2) only samples in the northern clade; and 3) only samples in the southern clade. I centered, but did not scale, variables and plotted the first two principal components for each analysis.

2.2.5 D-statistics

I used the comparison of full and censored topologies, phylogenetic networks, and PCAs to identify lineages demonstrating preliminary evidence of reticulate evolutionary histories. I then used D-statistics [88] to test for the presence of introgressed alleles in these candidate lineages. These tests require a four-taxon, pectinate phylogeny with the topology (((Taxon 1, Taxon 2), Taxon 3), Taxon 4) and test for differential introgression between Taxon 3 and Taxon 1 vs. Taxon 2. Here, I used D-statistics to evaluate introgression among four major lineages (i.e., Lineages S, E2, I, and J) and the major geographic clade (i.e., northern and southern clades) to which each does not belong (e.g., Lineage S and the southern clade). In each case, I created tests to compare the candidate lineage with its closest relative (that was not also implicated in a reticulate evolutionary history) as Taxon 1 and Taxon 2 [Table 2]. To select Taxon 2, I used the censored topologies I inferred from *north* and *south* assemblies [Results; Figure 4]. To avoid complexities created by bidirectional introgression, I used members of the southern and northern clades most geographically distant from sympatry between the two clades as Taxon 3. For the northern clade, I used Lineages A+B; for the southern clade, I used subset of Lineages K+L (i.e., E040, E060, E095, E050, E039, E124, E123, E122, E121) hereafter referred to as "Lineages K+L*". I used all outgroups and *E. wallacei* as Taxon 4. For each test, I used all SNPs from the *full_min5* assembly (i.e., a modified .loci output file from `ipyrad`) and conducted these tests using 100 bootstrap replicates in `PyRAD` v3.0.66 [47]. I used the generated Z-scores to determine statistical significance and plotted the densities of bootstrap replicates in `R` v3.5.1 [144].

2.2.6 Migration models using coalescent simulations

To further evaluate evidence of reticulate evolutionary histories among a subset of these same candidate lineages, I fit migration models to the multidimensional site-frequency spectrum (MSFS) using coalescent simulations in `fastsimcoal2`. [52]. I evaluated models for two sets

of four taxa: 1) Lineages E2, E1, A+B, and J; 2) Lineages G+H, C+F, A+B, and K+L* [see Results]. For each set of taxa, I evaluated six models consisting of all combinations of the following two specifications: 1) full vs. censored pectinate topologies recovered in phylogenetic analyses [Results; Figure 4]; and 2) no migration vs. unidirectional migration from Taxon 1 to Taxon 4 vs. bidirectional migration between Taxon 1 and Taxon 4. It is worth clarifying that because these simulated migration events occur in a coalescent framework, migration from Taxon 1 to Taxon 4 in the models corresponds to the more intuitive migration from Taxon 4 to Taxon 1 in forward-time.

I ignored monomorphic sites, and to calibrate the simulations, I fixed the final coalescent time to 3,400,000 generations based on estimates of the most recent common ancestor of the *E. bislineata* species complex (17 mya) [207] and a mean generation time of approximately five years [19]. To reduce model complexity, I estimated a single population size to be fixed across all populations and across time, estimated individual migration events, rather than continuous migration rates, and I constrained bidirectional migration events to occur simultaneously. For each set of taxa, I first calculated the MSFS from a dataset of all SNPs from the *full_min5* assembly (i.e., the .vcf output file from `ipyrad`) using `easySFS` [136]. To account for missing data, I pooled samples within lineages and used `easySFS` to "project down" to smaller sample sizes, balancing the number of loci with the number of samples. In `fastsimcoal2`, I sampled coalescent time parameters from a bounded uniform distribution of 10–3,400,000, population size parameters from an unbounded uniform distribution from 10–10,000,000, and all migration weights from bounded uniform distributions from 1 x 10^{-6}–0.99. I performed 40 ECM cycles and 200,000 simulations per run, and for each set of taxa, I compared models using AIC.

2.3 Results

2.3.1 Phylogenetic analyses

After quality-filtering, I recovered between 182,212 and 20,589,823 (median = 1,137,713) paired-end reads per sample. I exported the following assemblies: *full_min5* (192,046 loci;

2,053,633 SNPs), *full_min30* (23,808 loci; 545,180 SNPs), *north* (97,980 loci; 1,142,646 SNPs), and *south* (115,681 loci; 1,275,564 SNPs).

In all maximum-likelihood phylogenies, I recovered *E. [Haideotriton] wallacei* as sister to the *E. bislineata* species complex [Figure 4; Figure S2]. In the phylogenies inferred from the *full_min5* and *full_min30* assemblies, I recovered the monophyly of most major mtDNA lineages from Kozak et al. [106]. A noteworthy exception is Lineage E, which is paraphyletic; I will hereafter refer to the constituent clades as Lineages E1 and E2. Lineage K—which includes near topotypic samples for *E. cirrigera*—renders Lineage L paraphyletic, so I will hereafter refer to these two lineages together as Lineages K+L. Because I incorporated additional samples not included in Kozak et al. [106] (and thus do not have mtDNA data for all samples), it is not strictly possible to evaluate the monophyly of some other major mtDNA lineages. For this reason, I will hereafter primarily refer to Lineages G+H and Lineages C+F, rather than to their constituent mtDNA lineages. Because my sampling of Lineage A and Lineage B was limited, I will likewise refer to these two lineages together as Lineages A+B. Other notable examples of paraphyly result from E025 [see Discussion]; I recovered this sample as sister to Lineage M and Lineages K+L in the *full_min5* phylogeny and as sister to Lineage J in the *full_min30* and *south* phylogenies.

In phylogenies inferred from *full_min5* and *full_min30* assemblies, I found strong support for the existence of two major clades within the *E. bislineata* species complex, mostly corresponding to the northern and southern clades defined by mtDNA data in Kozak et al. [106]. However, I recovered the following alternative relationships: 1) Lineage I belongs to the northern clade; 2) Lineages G+H belong to the northern clade; and 3) Lineage F belongs to the northern clade. For the remainder of this study, I continue to use "northern" and "southern" clades to describe these groups, now referring to these new relationships rather than those from Kozak et al. [106]. Within the northern and southern clades, major mtDNA lineages corresponded well with river drainage boundaries, similar to Kozak et al. [106].

There are several topological incongruities between maximum-likelihood phylogenies inferred from uncensored, full assemblies (i.e., *full_min5* and *full_min30*) and those inferred from the censored *north* and *south* assemblies [Figure 4]. The topologies inferred from the censored datasets were significantly better than the topologies from the full dataset when

the censored datasets were used (north: Δ likelihood = -412.0, $p < 0.05$; south: Δ likelihood = -329.3, $p < 0.05$) [Table 1]. Likewise, the topology inferred from the full dataset was significantly better than the topology from the censored datasets when the full dataset was used (Δ likelihood = -2443.9, $p < 0.05$) [Table 1]. Most notable among the differences in the topologies are the placements of Lineages S, I, and E2, which are sequentially sister to the remainder of the northern clade in the full *full_min5* phylogeny but together form a clade that is sister to Lineage E1 in the censored *north* phylogeny. A similar pattern also exists for Lineages G+H, which are sister to Lineages C+F in the *north* phylogeny, and Lineage J, which is nested within Lineage M in the *south* phylogeny. These topological incongruities are consistent with expectations if these lineages have introgressed alleles from the major clade to which they do not belong (e.g., alleles from the southern clade in Lineage S).

In the *full_min30* phylogenetic network, I likewise recovered the major northern and southern clades, and relationships within these groups largely reflect those recovered in the maximum-likelihood phylogenetic trees [Figure 5; Figure S3]. Notably, network connections between Lineages S, I, and E2 and Lineage J and between Lineages G+H and the southern clade provide additional hints of introgression among these lineages. The networks including only samples from either the northern or southern clades largely reflect the relationships in ML phylogenies inferred from the censored *north* and *south* assemblies, respectively.

2.3.2 Principal components analyses

In the PCA including all samples from the *E. bislineata* species complex, the first principal component (PC1) explained 6.5% of variation, and PC2 explained 5.9% of variation [Figure 6; Figure S4]. The northern and southern clades separate on PC1, with PC2 primarily separating Lineages K+L from the remainder of samples. Notably, many samples from lineages identified as candidates for reticulate evolutionary histories in phylogenetic trees and networks (i.e., Lineages S, I, E2, G+H, and J) have intermediate scores on PC1, reinforcing the evidence that these groups have introgressed alleles from their distant relatives in the southern and northern clades, respectively. The PCAs including only samples from either the northern or southern clades largely reflect the relationships in ML phylogenies inferred from the censored *north* and *south* assemblies, respectively.

2.3.3 D-statistics

All D-statistics were significantly negative ($p < 0.05$), indicating evidence for differential introgression between the candidate lineages and southern or northern clades, respectively [Figure 7; Table 2]. Among tests focused on lineages from the northern clade, the most negative estimates were for tests including Lineage S (-0.23), then Lineage E2 (-0.14), then Lineage I (-0.10), then Lineages G+H (-0.06), an order which is congruent with the sequentially sister relationships inferred in the uncensored ML phylogenies. The estimate for the only test focused on a a lineage from the southern clade, Lineage J, was -0.13.

2.3.4 Migration models using coalescent simulations

For each set of taxa, the best model used the ML topology inferred from the censored assemblies. For the set of taxa including Lineage E2, the best model allowed for bidirectional migration between Lineages E2 and J. For the set of taxa including Lineages G+H, the best model allowed for unidirectional migration from Lineages G+H to Lineages K+L* (i.e., in coalescent terms; thus, this refers to migration from Lineages K+L* to Lineages G+H in forward time). In the former model, the migration estimate from Lineage E2 to Lineage J was 0.36, and the migration estimate from Lineage J to Lineage E2 was 0.17. The latter model, the migration estimate from Lineages G+H to Lineages K+L* was 0.04 [Figure 8; Table 3]. The relative magnitude of these inferred migration events is congruent with the order of sequential sister relationships in the full ML phylogeny and the relative magnitude of the D-statistics.

2.4 Discussion

2.4.1 Evolutionary history of the *E. bislineata* species complex

These complementary analyses provide consistent evidence for: 1) the paraphyly of four of five taxa, as currently defined, in the *Eurycea bislineata* species complex; 2) the existence of two major (northern and southern) clades; 3) the monophyly of most major mtDNA lineages identified in Kozak et al. [106]; 4) the coincidence of lineage boundaries with paleodrainage

divides; and 5) the role of widespread, ancient hybridization in structuring the current distribution of genetic diversity in the group, especially in regions with a dynamic history of river drainage reorganization.

All ML phylogenies recovered *E. [Haideotriton] wallacei*—a morphologically and ecologically distinct subterranean form restricted to the Floridan aquifer—as sister to the *E. bislineata* species complex, in contrast with previous studies that recovered it as nested within this group [207, 15]. The meaning and utility of monophyly and paraphyly are obscured by reticulate evolutionary histories, but most currently recognized species in the *E. bislineata* species complex do not meet any reasonable criteria for being recognized as such. The holotype of *Eurycea bislineata* likely belongs to Lineage B, but as currently defined, this name applies to Lineages A, B, and E1 and is paraphyletic. The holotype of *E. wilderae* likely belongs to Lineage E2, but as currently defined, this name applies to Lineage E2, M, and (parts of) J and is paraphyletic. The holotype of *E. cirrigera* probably belongs to Lineage K, but as currently defined, this name applies to Lineages C, D, F, I, K, and L and is paraphyletic. *E. junaluska* is described from only a small area, and thus, appears monophyletic, but it renders *E. aquatica* sensu lato paraphyletic. Finally, the "Sandhills *Eurycea*" has never been formally described, but is certainly distinct from the sympatric Lineage J. Because available names do not reflect the evolutionary history of the *E. bislineata* species complex, thorough taxonomic revisions are warranted.

All ML phylogenies, distance-based phylogenetic networks, and principal components analyses demonstrate evidence of two major clades (i.e., northern and southern) within the *E. bislineata* species complex, partially corroborating previous work using allozyme [91] and mtDNA sequence data [106]. Although Lineages G+H and Lineage I were relegated to the southern clade using mtDNA data, allozyme data suggested closer relationships to members of the northern clade. These general results are also congruent with secondary sexual characters; with the exception of the polymorphic Lineages E, M, and J, members of the northern clade have seasonally hypertrophied jaw musculature and small or absent cirri, while members of the southern clade have have elongate cirri and lack enlarged jaw musculature [Chapter 4].

While the coincidence between lineage boundaries and paleodrainage divides is evident in Kozak et al. [106] and in the *full_min5* and *full_min30* phylogenies and networks presented here, it is perhaps most striking in the *north* phylogeny [Figure 3; Figure 4]. There, I recovered three major groups of lineages largely corresponding to hypothesized paleodrainage basins: 1) the Old Ohio, Cumberland, Tennessee Rivers (Lineages G+H, Lineages C+F, and Lineage D); 2) the Teays River (Lineages E1, E2, I and S); and 3) the Laurentian River (Lineages A+B). Similarly, in the *south* phylogeny, I recovered a group of lineages (Lineages K+L and M) with distributions corresponding to with the Appalachian River paleodrainage. These topologies are consistent with initial, incipient speciation in allopatric river drainages and a partial reshuffling of genetic diversity following river drainage reorganization [106]. This is particularly true in the regions surrounding the two aforementioned paleodrainage hypotheses, which I will discuss in more detail below.

2.4.2 Reorganization of White's River

All phylogenies and networks including only taxa from the northern clade suggest the monophyly of a group (Lineages E1, E2, I, and S) distributed primarily in drainages associated with or adjacent to the Teays River paleodrainage. Lineage E1 is mostly found in the lower New–Kanawha and James River drainages—a phylogeographic pattern observed in other aquatic taxa and often explained by headwater captures by the James River [152, 80]. Lineage E2 is found primarily in the upper New River drainage, a region of high endemism in other aquatic organisms [81, 80]—but also in the neighboring headwaters of the Catawba and Yadkin–Peedee River drainages. Its close relative is Lineage I, which is distributed mostly in the Roanoke River drainage. This again is consistent with previous phylogeographic studies, which have invoked headwater captured by the Roanoke River to explain similarities in aquatic fauna among it and the New River [152, 80]. Finally, Lineage S occurs in small portions of the Cape Fear and Yadkin–Peedee River drainages in the Sandhills ecoregion of North Carolina. Lineage J occurs through most of the Atlantic Slope drainages in the region—including the Yadkin–Peedee and Catawba—but also in the French Broad and Nolichucky River drainages, which belong to the greater Tennessee River drainage. As Kozak et al. [106] suggested, this lineage may have originated in the highlands and later dispersed to

the Atlantic Slope. This hypothesis is consistent with other studies suggesting the headwater captures of the Tennessee River drainage by Atlantic Slope drainages [145, 172].

Generally speaking, these distributions are consistent with the existence of White's River. In particular, the occurrence of Lineage E2 in the headwaters of the Catawba and Yadkin–Peedee suggests a former connection between these and other streams hosting its close relatives, including the New and Roanoke River drainages. If this connection was White's River, the current distribution of Lineage S may reflect downstream dispersal following capture of its headwaters by the Yadkin–Peedee River. The potential intractability of the exact history of these events is underscored by sample E064, which I recovered in some analyses as belonging to Lineage E2, in others as sister to Lineage S, and which Kozak et al. [106] recovered as within Lineage I. Its membership to this larger group is not in doubt, but topological incongruities may be a signature of repeated hybridization.

The geographic proximity of these two distantly related groups—Lineage J and Lineages E2, I, and S—and the numerous river drainage reorganization events in the region likely created many opportunities for secondary contact, and the results from all analyses presented here suggest that introgression among these lineages has produced a complex, reticulate evolutionary history. In comparison to Lineage E1, Lineages E2, I, and S all show evidence of introgression from the southern clade, likely from Lineage J. There is some additional evidence that Lineage J is the donor of these alleles, as it is sister to Lineage I in the mtDNA phylogeny of Kozak et al. [106]. Similarly, all analyses demonstrate the presence of introgressed alleles from the northern clade in Lineage J. This suggests bidirectional gene flow between these members of the northern and southern clades, a hypothesis supported by the best-fit model in `fastsimcoal2`. It is possible that the morphological similarities between populations of Lineage E2 and J described as *E. wilderae* are attributable to this ancient hybridization and introgression.

Rather than reflecting few, discrete stream captures, the modern distribution of genetic diversity in this region may be the result of many such events. Variation in the proportion of the genome that is introgressed from the southern clade in Lineages E2, I, and S—as demonstrated in the sequential sister relationships in the *full_min5* phylogeny, the *full_min30* phylogenetic network, and variation in D-statistics—may provide some evidence for this

hypothesis. Furthermore, samples from Lineage E2 (E064) and Lineage S (E120) occur the furthest downstream in the Catawba and Yadkin–Peedee drainages, respectively, and show the strongest signal of introgressed alleles. Thus, these patterns may be most consistent with repeated or prolonged bouts gene flow between northern and southern clades. It is worth noting that today, Lineage S occurs in sympatry with Lineage J, and in at least one stream, Lineage E2 occurs in sympatry with Lineage M—another member of the southern clade. Although neither contact zone has been well-studied, there has been no suggestion of ongoing hybridization.

2.4.3 Formation of the modern Tennessee River

The lower portion of the hypothesized Appalachian River paleodrainage includes much of the distribution of Lineages K+L, and the upper portion includes the distribution of Lineage M. These lineages appear to be sister groups in some analyses (e.g., the *full_min5* phylogeny), but not others (e.g., the *south* phylogeny), potentially due to confounding intraclade introgression with Lineage J [see Discussion below]. The upper portion of the Appalachian River paleodrainage encompasses the only location of widespread sympatry between the northern and southern clades. Here, Lineage M is found in the highlands of the Cumberland Plateau and Blue Ridge. Lineages G+H—which belong to the northern clade and are sister to Lineages C+F in the Old Cumberland and Old Tennessee paleodrainages—are found in lowland springs and streams the Ridge and Valley and in mid-elevation streams in the Blue Ridge. At intermediate elevations and ecotones, Lineages G+H and Lineage M (or Lineages K+L) can occur in sympatry [164, 182]. In some locations (e.g., the distribution of *E. junaluska*), the two occur in syntopy and in reproductive isolation [164].

One explanation for the origin of this widespread sympatry is the formation of the modern Tennessee River River through the capture of the upper Appalachian River. Under this scenario, this vicariance was responsible for the divergence of Lineages M and L and the dispersal of Lineages G+H from the Old Tennessee to what became the Upper Tennessee River drainage. Alternatively, Kozak et al. [106] suggested that because the formation of the modern Tennessee River via the capture of the Appalachian River is congruent with estimated divergence times between Lineages M and L and between Lineages G and H, it

may have been responsible for vicariance and subsequent allopatric speciation in both pairs of putative sister taxa. This necessitates the preexisting sympatry between Lineages M and G and Lineages L and H, which could in turn be explained by headwater exchange between the Cumberland and Tennessee River drainages [29, 151, 172]—a hypothesis supported by the biogeography of numerous aquatic taxa [94, 79].

I found small, but significant, signals of introgression from the southern clade into Lineages G+H, consistent with the hypothesis of gene flow following river drainage reorganization and secondary contact. These results likely explain why the mtDNA phylogeny from Kozak et al. [106] placed these taxa in the southern clade—a relationship inconsistent with gross morphological data [160]. Today, Lineages G+H occur in sympatry with Lineages M and L, but their mtDNA haplotypes (curiously, along with Lineage F) are most closely related to those from Lineages I and J.

2.4.4 Additional secondary contact and introgression

Other examples of secondary contact and introgression are likewise suggestive of the influence of river drainage reorganization. One compelling example is sample E025, which sits near the divide between the Pigeon River—occupied primarily by Lineage M—and the French Broad River—occupied primarily by Lineage J. I recovered this sample as sister to Lineages L and M in the *full_min5* phylogeny, presumably due to alleles shared with Lineage J. This is corroborated by the *south* phylogeny, where I recovered E025 as sister to Lineage J, and the phylogenetic networks, which placed E025 between Lineages J and M. A sample from a nearby locality (E026) showed some similar patterns. In contrast to other examples presented here, this may represent the result of ongoing gene flow among these closely related lineages, but further work is necessary to test this hypothesis.

Kozak et al. [106] demonstrated the sympatry of Lineage E2 and Lineage M at one locality in the upper Linville River drainage of western North Carolina. This is the location of a smaller hypothesized drainage reorganization event, in which the Linville River (Catawba River drainage) captured its headwaters from the upper Toe River (Tennessee River drainage) [195]. One estimate of the timing of this event places it between the late Jurassic and Cretaceous [101]—far too early to be relevant for the sympatry of these two lineages of

Eurycea—but this is inconsistent with biological data suggesting a more recent date [145, 180]. I included the samples from Kozak et al. [106] in this study (Lineage E2: E097, E098; Lineage M: E102), and I recovered the same lineage assignments for each, with no evidence of additional introgression into Lineage E2 samples. Thus, although the genomes of Lineage E2 include introgressed alleles from the southern clade, the two appear to be reproductively isolated at this modern contact zone.

Lineages M and L occur in sympatry in several river drainages across the Appalachian foothills [27, 107]. These contact zones are near, but not perfectly aligned with, river drainage divides and may reflect the result of gradual drainage migration or an ecological determination of species distributions [Chapter 3]. It is noteworthy that these two lineages also occur in sympatry in the Hiwassee River of eastern Tennessee, as faunal similarities between this river and the Coosa have led to previous speculation about headwater exchange between them [104, 151]. Phylogenetic networks and PCAs suggest that at least some populations of these two lineages have experienced historic hybridization, although they appear to be reproductively isolated today [Chapter 3].

Finally, the distributions of Lineages A+B and D, and to some extent, Lineage E, in the northern clade occur largely within regions affected by Pleistocene glaciation, which dramatically reorganizated river drainages in the region [125, 5]. Kozak et al. [106] interpreted the deep divergence between Lineage D and Lineage E—despite both occurring in the modern Ohio River drainage—as evidence of the importance of Miocene and Pliocene paleodrainages, as these would have been situated in the Old Ohio and Teays River drainages, respectively. Consistent with this conclusion, I recovered the deep divergence between groups of lineages in the former Old Ohio, Cumberland, and Tennessee River paleodrainages and the Teays River paleodrainage.

2.4.5 Summary

Previous studies showed the importance of river drainage organization for the diversification of the *E. bislineata* species complex [106], but discordant phylogenetic relationships inferred from morphological, allozyme, and mtDNA datasets prevented a thorough understanding of

the evolutionary history of the group. Here, I used genome-scale data and a suite of phylogenomic methods to demonstrate several examples of ancient introgressive hybridization, revealing a reticulate evolutionary history in the *E. bislineata* species complex not adequately described by a bifurcating phylogeny. These new analyses explain incongruities among relationships suggested by earlier studies, including the relationships among populations currently identified as *E. wilderae* and the placement of *E. aquatica* and *E. junaluska*.

Furthermore, these results corroborate and expand upon previous work suggesting the role of paleodrainage reorganization in structuring the modern distribution of genetic variation. In particular, I demonstrated ubiquitous interclade introgression in regions with dynamic histories of drainage reorganization, revealing the importance of these large-scale events for initiating secondary contact between previously independently evolving lineages. However, my ability to make strong inferences about particular phylogeographic histories is limited for at least two reasons. First, the hydrogeological history the region is still contentious, and biological and geological evidence do not provide unanimous support for the hypotheses described here. New methods for modeling landscape evolution in the region will surely refine our understanding of this history, and biological hypotheses should be reevaluated accordingly [203, 54]. Second, these salamanders are not fully aquatic, and adults are capable of overland movements. Because gene flow may not be strictly confined to stream channels and may occur continuously across river drainage divides, drainage organization is unlikely to be the sole predictor of phylogeography. Future studies that use denser sampling and more mechanistic models of river drainage reorganization in regions of high lineage diversity (e.g., where the headwaters of the Tennessee, New, Catawba, and Yadkin–Peedee drainages meet in western North Carolina) may be able to disentangle these processes and provide answers at a finer resolution.

More broadly, this study adds to the growing body of literature demonstrating the ubiquity of reticulate evolution across diverse organisms and emphasizing the importance of accounting for introgression when inferring these evolutionary histories [179]. The phylogeography of stream-dwelling organisms make them particularly compelling systems for studying these processes [54], and as methods for modeling the evolution of both rivers

and their inhabitants improve, so too will the understanding of the processes that generate and maintain diversity.

Chapter 3

Fine-scale ecological segregation in replicate contact zones

3.1 Introduction

Genomic data have revealed the ubiquity of reticulate evolutionary histories [179], and evidence of ancient hybridization between species currently in allopatry implies previous periods of secondary contact [23]. Although the processes structuring gene flow at ancient contact zones may be difficult to resolve, modern contact zones provide an opportunity to study them directly. In general, secondary contact can result in either: 1) hybridization; 2) maintenance of species boundaries and sympatry, enabled by reproductive isolation and ecological divergence; 3) competitive exclusion of one species; or 4) some combination of these outcomes. Thus, while large-scale geologic or climatic change may be responsible for initiating secondary contact [76], behavioral and ecological factors may be important for determining the distribution of genes and species through hybridization and competition.

In the *E. bislineata* species complex, phylogeographic analyses demonstrate a strong correspondence between paleodrainage divides and putative species boundaries, with river drainage reorganization events (e.g., stream capture) initiating secondary contact between previously independently evolving lineages [Chapter 2]. Although evidence for widespread, ancient introgression among the major northern and southern clades suggests an absence of reproductive isolating mechanisms in the early evolutionary history of the group, modern

contact zones—both between and within the northern and southern clades—reveal a broader diversity of outcomes.

3.1.1 Contact zones between northern and southern clades

At least eight examples of modern secondary contact have been described in the *E. bislineata* species complex. The clearest example of widespread sympatry between two species is between one member of the northern clade (*E. junaluska*; Lineage G in Kozak et al. [106]) and one member of the southern clade *E.* cf. *wilderae*; Lineage M) in the Blue Ridge. In this example, the two species are easily morphologically distinguishable and markedly ecologically divergent, with Lineage G found only in large, lower-gradient streams and Lineage M found primarily in small, high-gradient streams [138, 164]. Other examples of contact zones and limited sympatry between northern and southern clades include Lineage H and Lineage L in northern Alabama [100, 182, Pierson, pers. obs], Lineage F and Lineage M on the Cumberland Plateau of Tennessee [Pierson, pers. obs.], Lineage E2 and M in western North Carolina [106], and Lineage S and J in central North Carolina [13]. In most of these examples, the two species also appear to be morphologically distinguishable and ecologically divergent. Although few have been studied in sufficient detail to make strong conclusions, none of these contemporary contact zones show evidence of ongoing hybridization, suggesting modern reproductive isolation between northern and southern clades.

3.1.2 Contact zones within northern or southern clades

In contrast, contact zones within these major clades have most often been characterized by ongoing, if limited, hybridization. Guttman and Karlin [65] described a contact zone between two members of the northern clade (most likely Lineages B and D) in central Ohio, which may have originated relatively recently following river drainage reorganization during Pleistocene glaciations [125, 5, 106]. These authors used allozyme data to argue for limited gene flow in a narrow hybrid zone. Similarly, Ghitea and Sattler [55] found morphological and allozyme evidence for limited gene flow in a narrow hybrid zone between another pair of lineages from the northern clade (Lineages A+B and E1) in Virginia. Finally, Miller

and Hallerman [126] used allozyme data to examine the contact zone between sister taxa—Lineage A and Lineage B—in Maryland and demonstrated evidence of widespread gene flow. In all of these examples, reproductive isolation is weak, morphological differences are subtle and inconsistent, and there is no evidence of substantial ecological divergence.

The most thoroughly examined contact zones occur between two lineages in the southern clade (*Eurycea cirrigera*; Lineage L and *Eurycea* cf. *wilderae* Lineage M) in the foothills of the southern Appalachians. Camp et al. [27] first described sympatry between these species in northern Georgia, using morphological and allozyme data to argue for very limited or nonexistent gene flow. In the same region, Marshall [121] interpreted allozyme, mtDNA, and morphological data as evidence of variation in the prevalence of introgression among replicate contact zones. Across a larger, parallel transect in North and South Carolina, Kozak and Montanucci [107] found evidence for limited gene flow between the two species, again using allozyme data. The possibility of gene flow between these species was reinforced by Kozak [105], who used courtship trials to demonstrate only weak sexual isolation—evidence that a strong prezygotic reproductive barrier may be lacking. Collectively, these studies suggest ongoing, but limited, gene flow between Lineages L and M, which is consistent with phylogenomic data that show evidence of at least some ancient hybridization and introgression between these lineages [Chapter 2].

Notably, Lineages L and M are ecologically divergent in allopatry and in sympatry in at least three ways. Camp et al. [27] reported that within contact zones, Lineage L alone is found in large, low-gradient streams, Lineage M alone is found in small, high-gradient streams, and the two occur in sympatry in intermediate habitats. These ecological differences recapitulate the broader distributions of each species, as Lineage L is distributed primarily through lowland habitats in the Piedmont and Coastal Plains, while Lineage M occurs in upland habitats of the Appalachians highlands [138]. Where they are sympatric, differences in nest site selection may cause fine-scale spatial segregation between these species. At one such site where gene flow between species appears limited, Marshall [121] demonstrated that Lineage L selects larger rocks (probably characteristic of stream pools) and Lineage M selects smaller rocks (probably characteristic of riffles). In contrast, at a site with greater putative gene flow, there was no difference among nesting sites. This led Marshall [121] to speculate that

hybridization may be more common in areas with greater ecological intermediacy. Although adults (and thus, nesting sites) appear to segregate along an ecological gradient in contact zones, it is unclear whether the same is true for aquatic larvae. Strickland et al. [176] demonstrated that larvae of these two species have different thermal preferences, but because animals were collected from allopatric sites, it is unclear whether these differences results from ecological divergence among species or local adaptation. Finally, evidence of ecological divergence can also be gleaned from differences in body size. In allopatry, Lineage M is slightly smaller than Lineage L—potential evidence of differences in fundamental ecological niche. In sympatry, Lineage L is significantly larger than in allopatric populations, and thus, the difference between species is greater—a phenomenon suggestive of character displacement through competition [27, 121].

Wray and Steppan [207] argued that lability of ecological niche drove early diversification in the tribe Spelerpini, but that this was followed by "subsequent vicariance with *in situ* diversification" in *Eurycea*. This is consistent the results of phylogeographic studies of the *E. bislineata* species complex, which demonstrate allopatric speciation driven by the organization of river drainages [Chapter 2; 106]. Because other groups of plethodontid salamanders with similar evolutionary histories show patterns of ecological niche conservatism, we may expect the same in the *E. bislineata* species. However, as the examples described above have illustrated, this is not universally true. In several zones of secondary contact, species show marked ecological differences, and in at least one—between Lineage L and M—these differences are implicated in structuring gene flow.

3.1.3 Objectives

Here, I collected and analyzed paired genomic and environmental data from four replicate contact zones to reevaluate patterns of reproductive isolation, hybridization, and ecological divergence between Lineages L and M. More specifically, my objectives were to: 1) estimate variation in introgression among contact zones; and 2) define the role of fine-scale environmental factors in structuring genetic variation.

3.2 Methods

3.2.1 Sampling and environmental data collection

I selected four replicate contact zones (hereafter Sites 1–4) between *E. cirrigera* (Lineage L) and *E.* cf. *wilderae* (Lineage M) in the Appalachian foothills of northeastern Georgia [Figure 9]. Each of these sites is the location of confirmed sympatry, and several have previously served as study sites for research focused on secondary contact between these two species [27], with Marshall [121] suggesting variable levels of introgression among them.

At each site, I established a stream transect in the reach of the stream with previously confirmed sympatry. At Sites 2–4, these transects were uninterrupted 500 m stream-lengths. At Site 1, this transect consisted of two 75 m sections situated immediately downstream and upstream of an approximately 300 m stream reach that is redirected into underground culverts below a baseball field. Within each transect, I sampled approximately every 5 m, noting the exact location of the sample in the transect to the nearest meter. At each sampling point, I searched among aquatic leaf-litter and cobble and collected one nonlethal tissue sample from a first-year *E. bislineata* species complex larva; at this stage, the two sympatric species are morphologically indistinguishable. Paired with each tissue, I also collected the following stream characteristics: maximum stream depth and stream width (to the nearest cm), stream velocity (measured in sec/m using a ping-pong ball and stopwatch and converted to m/sec), and stream habitat type (i.e., pool, run, or riffle). I stored tissues in 95% EtOH at -20°C until extraction.

3.2.2 RADcap bait design

To evaluate gene flow between species, I used RADcap data [83]. In brief, this method uses 3RAD libraries prepared from a small number of samples for SNP discovery and uses capture baits to consistently recover a subset of those SNPs in a larger number of samples. This method is particularly appealing for projects involving many samples from organisms with large genomes (e.g., salamanders), as the generation of datasets with low amounts of missing data can otherwise be costly [197]. First, to design RADcap baits, I generated 3RAD [11]

libraries for five individuals from Lineage L and five individuals from Lineage M collected from outside of the contact zone between the two species (E011, E033, E041, E054, E061, E074, E075, E090, E093, E094; Table S1). I used the enzymes XbaI, NheI-HF, and EcoRI-HF and followed the library preparation protocol detailed in Bayona-Vásquez et al. [11]. I size-selected these libraries for 500 bp +/- 10% using a Pippin Prep, pooled them with libraries from unrelated projects, and sequenced them on an Illumina NextSeq PE150 run.

I demultiplexed all reads by external indexes (i.e., iTru7/iTru5) [Table S1] using `bcl2fastq` (Illumina, Inc.). I quality-filtered and assembled the reads *de novo* using `ipyrad` [47, 46], using a clustering threshold of 0.85 and filtering for loci that were present in at least seven samples. This produced 1,373 loci. Using the .loci output from `ipyrad`, I reformatted R1 and R2 consensus sequences into FASTA files and sent them to MYcroarray (now Chiral Technologies, Inc.) for bait design. Following their filtering criteria, I selected two baits each (i.e., one from R1 and one from R2) for 1,000 loci and ordered a 20,000 bait kit consisting of these 2,000 baits and 18,000 baits from unrelated projects. Finally, I created a FASTA file with these 1,000 loci to serve as a pseudo-reference genome for assembly of RADcap reads.

3.2.3 RADcap library preparation and sequencing

I extracted DNA from all 330 tissue samples using Qiagen DNeasy Blood and Tissue Kits, and I quantified DNA extracts using a Nanodrop Spectrophotometer. I normalized DNA extracts and followed the 3RAD protocol through the ligation step, using the internal indexes built into adapters to ensure that I could pool 66 samples per capture [11]. Following ligation, I created five pools, each consisting of 7 μL each of 66 samples. I cleaned these ligation products with a 1.2:1 SpeedBeads:DNA volume ratio and resuspended in 50 μL TLE.

I then followed the RADcap protocol as described in Hoffberg et al. [83], with small modifications. First, I conducted single-cycle, 8N PCRs to index individual molecules for downstream filtering of PCR duplicates. Using Kapa HiFi Hotstart reagents and the cleaned ligation product described above, I set up two reactions per pool using the following recipe: 15 μL DNA, 5 μL 8N primer, 10 μL buffer, 1.5 μL dNTPs, 17.5 μL H_2O, and 1 μL polymerase. I also set up two reactions per pool using the following, alternative recipe: 5 μL DNA, 5 μL 8N primer, 10 μL buffer, 1.5 μL dNTPs, 27.5 μL H_2O, and 1 μL polymerase. I conducted

PCRs using the following thermocycler conditions: 98°C for 40 sec; 98°C for 20 sec, 60°C for 30 sec, 72°C for 1 min; 72°C for 5 min. For each pool, I then combined and cleaned all PCR products using a 1.2:1 SpeedBeads:DNA volume ratio and resuspended in 30 μL TLE. Then, I conducted a limited-cycle PCR using these PCR products and the aforementioned PCR reagents. I conducted three reactions per pool, each with the following recipe: 10 μL DNA, 5 μL P5 primer, 5 μL iTru7 primer (with unique indexes per replicate), 10 μL buffer, 1.5 μL dNTPs, 17.5 μL H_2O, and 1 μL polymerase. I conducted PCRs using the following thermocycler conditions: 98°C for 40 sec; then 6 cycles of 98°C for 20 sec, 60°C for 30 sec, 72°C for 1 min; 72°C for 5 min. For each pool, I then combined and cleaned all PCR products using a 2:1 SpeedBeads:DNA volume ratio and resuspended in 40 μL TLE.

To validate libraries before capture reactions, I used a small aliquot of each these pools in test PCRs with the following recipe: 1 μL DNA, 2.5 μL P5 primer, 2.5 μL P7 primer, 5 μL buffer, 0.75 μL dNTPs, 12.75 μL H_2O, and 0.5 μL polymerase. I conducted PCRs using the following thermocycler conditions: 98°C for 40 sec; then 15 cycles of 98°C for 20 sec, 60°C for 30 sec, 72°C for 1 min; 72°C for 5 min. I ran this product on a 1.5% agarose gel, visualized a smear to validate the product, and discarded the product. I then quantified each of the five pools using a Qubit 2.0 Fluorometer, and the products varied between 21.4–26.1 ng/μL.

I conducted captures following the manufacturer's protocol, using a 65°C capture temperature and a 27-hour capture period. Following the wash and cleanup steps, for each of the five pools, I used 1/3 of the capture product volume to conduct PCRs with the following recipe: 10 μL DNA, 2.5 μL P5 primer, 2.5 μL P7 primer, 5 μL buffer, 0.75 μL dNTPs, 3.75 μL H_2O, and 0.5 μL polymerase. I conducted these PCRs using the following thermocycler conditions: 98°C for 40 sec; then 12 cycles of 98°C for 20 sec, 60°C for 30 sec, 72°C for 1 min; 72°C for 5 min. I then quantified these products on a Qubit 2.0 Fluorometer, and the products varied between 0.162–0.180 ng/μL. Because these concentrations were slightly lower than ideal, I conducted PCRs with the remainder of the capture products using the same recipe and thermalcycler protocol, except for 18 cycles, and I conducted an additional 6 cycles of PCR for the first PCR product. Then, for each original capture pool, I combined each of these PCR replicates, cleaned them using a 2:1 SpeedBeads:DNA volume ratio, and resuspended them in H_2O. I quantified each of the five pools using a Qubit 2.0 Fluorometer

and pooled them proportionately into one final pool. Leaving the SpeedBeads behind, I transferred the liquid to a new microcentrifuge tube and again quantified this pool again using the Qubit 2.0 Fluorometer (3.57 ng/μL). I pooled these libraries with unrelated projects and sequenced them on an Illumina HiSeq 4000 PE150 sequencing run.

3.2.4 RADcap data assembly

First, I demultiplexed reads by pool using the iTru7 index with the `process_radtags` program in `Stacks` v1.47 [30]. Next, also with `process_radtags`, I demultiplexed individual samples by internal indexes, and I removed PCR duplicates within each sample using the program `clone_filter`, also in `Stacks` v1.47. I assembled reads in `ipyrad` v0.7.19 [47, 46] against the pseudo-reference genome consisting of the 3RAD loci used to design baits; I used a clustering threshold of 0.85 and a minimum read depth of 6. I created and exported three assemblies: 1) *min5*, consisting of loci present in at least five individuals; 2) *min100*, consisting of loci present in at least 100 individuals; 1) *min250*, consisting of loci present in at least 250 individuals.

3.2.5 Population assignment using `Structure` and DAPC

To account for potential bias created by SNP selection and filtering criteria [117], I created the following four versions of each of the three aforementioned assemblies: 1) all SNPs; 2) all SNPs with a minor allele frequency (MAF) > 0.05; 3) one random SNP per locus; and 4) one random SNP per locus with MAF > 0.05. With each of these twelve datasets, I then used two methods to evaluate potential hybridization and between Lineages L and M.

First, I used the Bayesian clustering program `Structure` v2.3.4 [143]. Because I had *a priori* knowledge that these samples came from two populations (i.e., two species), I used K = 2. I sampled for 100,000 iterations following 10,000 burnin iterations. I conducted these analyses twice: once using the admixture model and once using the no-admixture model. To test whether allowing for admixture improved the fit of the model, I calculated Bayes factors (i.e., the ratio of the estimated marginal likelihoods of the admixture model and the no-admixture model) for each pair of models [53]. In general, Bayes factors of 20:1 can

be considered "strong evidence", and those over 100:1 can be considered "very strong or definitive evidence" for one model over the other [102, 56]. I then examined posterior mean estimates of admixture proportions and posterior probabilities of population assignment for the admixture and no-admixture models, respectively, to evaluate evidence of hybridization.

Because `Structure` results may be strongly affected by the MAF threshold [117], I also conducted discriminant analyses of principal components (DAPC) implemented in the package `adegenet` in `R` v3.5.1 [98, 144]. First, I used k-means clustering and examined Bayesian Information Criteria (BIC) to identify the number of clusters most appropriate for the data. Then, using these results (i.e., two clusters), I conducted a DAPC to obtain posterior membership probabilities for each individual. For each individual, I calculated the mean of posterior mean estimates of admixture proportions from `Structure` runs using the admixture model and mean posterior membership probabilities from DAPCs. To determine which modeled populations correspond to Lineage L and Lineage M, I compared allele frequencies from inferred populations to those from the reference sequences used to designed RADcap baits.

To characterize the SNPs used for these analyses, I used the dataset consisting of all SNPs from the *min5* assembly and the mean DAPC membership probabilities to calculate: 1) the percentage of all SNPs with MAF > 0.05; 2) the percentage of all SNPs that are biallelic; 3) the percentage of biallelic SNPs that are present in both species; 4) the percentage of biallelic SNPs that are present and polymorphic in both species; 5) the percentage of biallelic SNPs that are present in both species and that are diagnostic for species identity; and 6) using the package `hierfstat` [57], the distribution of locus-specific (i.e., SNP-specific) F_{ST}. I conducted all of these analyses in `R` v3.5.1 [144].

3.2.6 Comparison of habitat characteristics

To evaluate whether subjective categorizations of stream habitats reflect quantitative stream measurements, I compared measurements for maximum depth, width, and velocity among pools, runs, and pools from all sites using ANOVA ($\alpha = 0.05$) in `R` v3.5.1 [144]. For measurements with significant differences among habitats, I then used Tukey tests to examine all pairwise differences ($\alpha = 0.05$).

3.2.7 Comparison of species composition among stream habitats

I assigned each of the 330 individuals to one of the two species based on `Structure` and DAPC results [see Results], and to compare the distribution of species among pools, runs, and riffles, I fit a binomial generalized linear mixed-effects model ($\alpha = 0.05$) using the package `lme4` in `R` v3.5.1 [10, 144]. I used stream position (i.e., distance from the beginning of the transect) and these stream habitat categories (setting "run" as the intercept) as fixed effects, and I used site as a random effect.

3.3 Results

3.3.1 Population assignment using `Structure` and DAPC

After quality-filtering, I recovered between 23,509 and 147,856 (median = 60,672) paired-end reads per sample. I exported the following assemblies: *min5* (730 loci; 9,211 SNPs), *min100* (466 loci; 6,005 SNPs), and *min250* (176 loci; 2,441 SNPs).

Means of posterior mean estimates of admixture proportions in `Structure` and mean posterior membership probabilities from the DAPC were consistent, and I hereafter refer to these simply as "assignments". In both sets of analyses, the same 44% of individuals received assignments of ≥ 0.99 to a Lineage L and the same 56% of samples received assignments of ≥ 0.99 to a population corresponding with Lineage M [Table S2; Figure 10]. All Bayes factors comparing admixture and no-admixture models in `Structure` were 1.0, suggesting that allowing for admixture did not improve the models. Thus, these results provide no evidence for ongoing gene flow between Lineages L and M.

In total, 36% of SNPs had MAF >0.05, and 91% of SNPs were biallelic. Of those biallelic SNPs, 84% were present in both species, 5% were polymorphic in both species, and 12% were diagnostic for species identity. The distribution of SNP-specific F_{ST} was strongly bimodal for approximately 0 and 1 [Figure S5].

3.3.2 Comparison of habitat characteristics

Stream habitats differed significantly in maximum stream depth ($F = 95.1$, $p < 0.01$) and velocity ($F = 125.1$; $p < 0.01$), but not width ($F = 2.2$; $p = 0.11$) [Figure 11]. As expected, maximum stream depth was greatest in pools, intermediate in runs, and smallest in riffles, and all pairwise differences were significant ($t = 11.1$, -4,9, 1.2; $p < 0.01$). Also as expected, velocity was slowest in pools, intermediate in runs, and fastest in riffles, and all pairwise differences were significant (t = -9.1, 10.0, 15.4; $p < 0.01$). These results confirm that categorical classifications of stream habitats correspond to expectations about quantitative habitat measurements.

3.3.3 Comparison of species composition among stream habitats

Averaged across all streams, 12% of individuals from pools (range = 0–19%), 52% of individuals from runs (range = 29–58%), and 83% of individuals from riffles (range = 77–100%) were assigned to Lineage M [Figure 10], with the remainder being assigned to Lineage L. Habitat was a strong predictor of species composition, as both pools (transformed coefficient = 0.18; $p < 0.01$) and riffles (transformed coefficient = 0.88; $p < 0.01$) were significantly different than the intercept (i.e., runs; transformed coefficient = 0.46). Stream position was not a significant predictor of species identity (transformed coefficient = 0.50; $p = 0.66$); although there is a correlation between stream position and habitat within streams, these correlations are inconsistent among the four sites [Figure S6]. These results demonstrate consistent differences in species composition among pools, riffles, and runs in replicate contact zones.

3.4 Discussion

The `Structure` and DAPC results provide no evidence for ongoing gene flow between these two closely related and sympatric species in the *E. bislineata* species complex. Many SNPs had low MAF and low F_{ST}, suggesting a high prevalence of recent, low-frequency variants and/or unfiltered sequencing errors. Still, the abundance of diagnostic SNPs is consistent

with no ongoing gene flow between species. This partially corroborates previous studies that used allozyme and mtDNA data to study contact zones between these same two species, including at some of the same sites [27, 121, 106]. However, some interpretations of those earlier data—and accompanying morphological data [121]—suggested low levels of gene flow, a conclusion inconsistent with the results of this study. In these earlier studies, few molecular markers were diagnostic at the species level, and similar allele frequencies and morphological intermediacy in contact zones may have reflected similar patterns of natural selection upon retained ancestral polymorphisms or alleles introgressed during ancient hybridization events.

All four contact zones were characterized by fine-scale ecological segregation between Lineage L and Lineage M, with the former more common in pools and the latter more common in riffles. These differences recapitulate the environmental differences from the allopatric portions of their distributions, where Lineage L is widespread throughout the Piedmont and Coastal Plains and Lineage M is found at higher elevations in the Blue Ridge and Cumberland Plateau, and they corroborate similar hypotheses described by previous studies [27, 121]. The qualitative and quantitative stream characteristics I measured describe the entire stream width at the point of capture. However, at a finer spatial scale, most salamander captures came from leaf litter along the stream periphery. It is likely that these microhabitats—found in parts of the stream characterized as pools, runs, and riffles—are more similar than my data suggest. Thus, it seems unlikely that the fine-scale ecological segregation by Lineages L and M is the result of active sorting by larvae, a conclusion consistent with behavioral experiments suggesting no differences in thermal preference between these species in sympatric populations [Camp, unpubl. data].

Alternatively, if Lineage L and M females nest primarily in pools and riffles [121], respectively, the spatial distribution of young larvae may simply reflect the legacy of these choices. It is noteworthy that I observed this fine-scale ecological segregation at Site 4, where Marshall [121] found no evidence for differences in nest site selection between species. However, Marshall [121] directly measured the size of rocks used for nests, rather than the stream habitat in which they were located. Thus, it is possible that his data reflect a poor correspondence between rock-size and stream habitat, rather than a breakdown in ecological segregation. If the distribution of larvae does reflect differences in nest site selection,

replication of my study with second-year larvae—which, unlike recent hatchlings, have had a chance to disperse downstream—should produce a more diffuse association between stream habitat and relative species composition. Additionally, replication of this study at other contact zones between these two species—especially in areas where previous phylogeographic research has suggested historic introgression—could reveal whether the outcomes I observed here are universal. There exists at least one additional, geographically disjunct contact zone between these species in Tennessee's Hiwassee River, where anecdotal evidence suggests similar fine-scale ecological segregation [Pierson, pers. obs.].

The origin and nature of reproductive isolation between Lineages L and M is unknown. Kozak [105] demonstrated some degree of assortative mating in laboratory trials between these species, but 26% of interspecific pairings still resulted in insemination. This suggests that divergent courtship behaviors or pheromones alone are not a sufficiently strong reproductive isolating mechanisms to explain the lack of gene flow I describe here. It also seems unlikely that ecological segregation within stream channels is a sufficient explanation, as courtship is largely synchronous and occurs primarily in shared terrestrial habitats [138]. Future studies exploring rates of fertilization following interspecific insemination, viability of eggs, and fitness of potential hybrid offspring may provide clearer answers.

In summary, these results demonstrate that although large-scale, hydrogeological changes drive diversification and initiate secondary contact in the *E. bislineata* species complex [Chapter 2], fine-scale, ecological factors may subsequently determine the distribution of genes and species. Despite the two lineages sharing a relatively recent common ancestor, contact zones between Lineages L and M reveal reproductive isolation and fine-scale ecological segregation. This is in contrast to contact zones between morphologically and ecologically similar forms, which are characterized by ongoing gene flow [65, 126]. The existence of sympatry between species pairs with variable degrees of molecular, morphological, and ecological divergence in the *E. bislineata* species complex creates a compelling system for future comparative studies of the influence of these factors on the outcome of secondary contact.

Chapter 4

Alternative reproductive tactics

Portions of this chapter have been published in The American Naturalist (© 2019) [141] or have been accepted for publication in Herpetological Review [140].

4.1 Introduction

Variation in breeding behavior and morphology is correlated with reproductive isolation in many organisms, including plethodontid salamanders [124, 86, 187, 188, 190]. The *Eurycea bislineata* species complex exhibits internal fertilization and uses primarily lotic nesting sites. In the breeding season, males develop a small, fan-shaped mental gland with a circular pad and accompanying premaxillary teeth that pierce the upper lip; these features are used in the production and delivery of courtship pheromones to females. Males of some species (e.g., *Eurycea cirrigera* sensu stricto) within this species complex develop elongate cirri during the breeding season, presumably to aid in the discovery of receptive females [43, 158, 161, 159]. These traits are similar to those shared among many other species in the genus *Eurycea* and its sister genus *Urspelerpes* [165, 26]. Males of other species (e.g., *E. aquatica*) either lack or under-develop these cirri but instead have seasonally hypertrophied jaw musculature [1, 59]. Previous systematic studies [91, 106] using allozyme and mitochondrial DNA sequence data, respectively, led Deitloff et al. [36] to conclude that species with each of these two forms do not constitute monophyletic groups; however, new phylogenomic data suggest that they likely do [Chapter 2].

Several reports of agonistic behavior in the *E. bislineata* species complex [6, 181] demonstrate that males may bite other males, especially in the reproductive season, but omit explicit comparisons between species. Deitloff et al. [36] demonstrated that male *E. aquatica* exhibit mate-guarding (as measured by biting, among other behaviors), but male *E. cirrigera* do not, showing that differences in secondary sexual characters correspond to differences in reproductive behavior. Across the *E. bislineata* species complex, the male form with elongate cirri, a mental gland, protruding premaxillary teeth, and lacking mate-guarding behavior has been described variably as "cirriferous", "cirrigerous", "cirri-possessing", "small-headed", and "mate-searching"; here, I refer to it as the searching morph. The other male form (i.e., with hypertrophied jaw musculature, a mental gland, protruding premaxillary teeth, and with mate-guarding behavior) has been described variably as "large-headed", "cirri-lacking", and "mate-guarding"; here, I refer to it as the guarding morph. With a key exception described below, all species in the *E. bislineata* species complex appear to have morphological and behavioral traits assignable to only one of these two distinct forms (searching: *E. cirrigera*; guarding: *Eurycea aquatica, E. junaluska, E. bislineata*, and *E.* cf. *cirrigera*).

Sever [160] described an apparent exception to this paradigm. In populations of *E. wilderae* from the Southern Appalachians traditionally considered to have the searching morph, some males instead exhibit a phenotypic suite more similar to the guarding morph. One important difference is that while these males have seasonally hypertrophied jaw musculature, they lack the mental gland and enlarged premaxillary teeth found in species with the guarding morph [Figure 12]. Sever [160] described these individuals as "Morph A" and indicated that although this form may represent an intraspecific polymorphism, he suspected that it represents a distinct, cryptic species sympatric with *E. wilderae*. Subsequent courtship trials have suggested small differences in the behaviors of these two morphs, but have found no evidence of reproductive isolation [147, 105]. Molecular systematic studies have found no evidence to support the hypothesis that Morph A represents a cryptic, sympatric species, but they have demonstrated that *E. wilderae* sensu lato is paraphyletic with respect to all other species in the *E. bislineata* species complex and consists of at least three putative species [Chapter 2; 91, 106]. The holotype of *E. wilderae* belongs

to one of these species—"Lineage E" in Kozak et al. [106]. However, the majority of the geographic distribution of *E. wilderae* sensu lato is occupied by two other putative species ("Lineage M" and "Lineage J"). Before the present study, Morph A was known conclusively from within the distribution of two of these putative species—Lineages M and J. Here, I include data from all three putative species, and while the broader evolutionary history of this group is beyond the scope of this publication, I will refer to these lineages either by their Kozak et al. [106] names or as *E. wilderae* (i.e., Lineage E) and *E.* cf. *wilderae* (i.e., Lineages M and J) to reduce confusion caused by a paraphyletic taxonomy.

Through several fall and winter months, Bruce [19] conducted a systematic census of *Eurycea* cf. *wilderae* in North Carolina, using a dipnet to survey only individuals within a small stream channel. He demonstrated that in October, the only adult salamanders in the stream were females and Morph A males, and later in the winter, the relative frequencies of Morph A and searching males grew closer to even. Like searching males, juveniles also appeared to migrate to this stream later in the season than females or Morph A males. Sever [160] likewise noted that at some localities, he found only Morph A males, while in others, he found only searching males. Upon revisiting some of these sites after the publication of the original data, he sometimes found only the other morph [Sever, unpublished data]. These data suggest the possibility of reproductive phenological differences between these two male forms during courtship and migration to breeding streams.

4.1.1 Objectives

Here, I collected and analyzed genomic, behavioral, karyological, and field observational data to address whether Morph A represents an ART found within populations *E. wilderae* and *E.* cf. *wilderae*. More specifically, I used genomic data to test whether Morph A males collected from different populations are monophyletic with respect to searching males from the same populations, directly testing whether this morphological variation is indicative of cryptic, sympatric species or an intraspecific polymorphism. I used behavioral trials to test whether Morph A males are more likely to exhibit mate-guarding behavior than are searching males, directly testing whether Morph A males share a reproductive tactic with guarding males in monomorphic species. ARTs in some organisms are structured by "supergenes"

originating as chromosomal inversions [e.g., 112, 184], and because a chromosomal inversion polymorphism is documented on chromosome 13 of *Eurycea* cf. *wilderae* [157], I used karyological data to test for a causal relationship between this inversion and male morph. Finally, I gathered field observational data to test for differences in the fine-scale distribution and reproductive phenology of these two forms, providing complementary data to evaluate whether the observations of Bruce [19] are replicable.

4.2 Methods

4.2.1 Phylogenetic analysis

To test whether Morph A represents a distinct, cryptic species, I used a phylogenetic approach. As a subset of a larger phylogenomic study [Chapter 2], I collected tissues and extracted DNA from sympatric Morph A and searching males in each of the three putative species (i.e., Lineages E, M, and J) comprising the paraphyletic *Eurycea wilderae*. For these six samples plus representative samples from across the *Eurycea bislineata* species complex, including one sample from near the type locality of *Eurycea wilderae* [Figure 13], I generated 3RAD libraries [11]. I prepared these libraries using the enzymes XbaI, NheI-HF, and EcoRI-HF, and I used a Pippin Prep to size-select for 500 bp +/- 10%. I pooled these libraries with those from unrelated projects and sequenced them on an Illumina NextSeq PE75 run at the Georgia Genomics Facility. I demultiplexed reads by external indexes (i.e., iTru7/iTru5) using `bcl2fastq` (Illumina, Inc.). I used `ipyrad` [47, 46] to filter, trim, and assemble reads *de novo*. I inferred a maximum-likelihood (ML) phylogeny from concatenated loci using `RAxML` v8.2.11 [171]. I conducted 100 rapid bootstrap replicates and a thorough ML search on the best tree using a GTRGAMMA substitution model. I rooted the phylogeny with the outgroups *E. guttolineata* and *E. longicauda*. I conducted all data processing and phylogenetic analyses on an Amazon EC2 Instance.

4.2.2 Behavioral trials

To examine whether this morphological polymorphism is correlated with a reproductive behavioral polymorphism, I conducted a series of behavioral trials in the laboratory following the methods of Deitloff et al. [36]. I collected 152 adult *E.* cf. *wilderae*, representing 48 Morph A males, 53 searching males, and 51 females from the Blue Ridge of Tennessee and North Carolina, representing both putative species—Lineages M and J—from which this potential polymorphism was previously known. I conducted 69 mate-guarding trials similar to Deitloff et al. [36]. Briefly, I placed a male *E.* cf. *wilderae* (i.e., the resident male) in a 16 x 16 x 5 cm plastic container covered with a plexiglass lid for 20 minutes. I then introduced a female to the enclosure for 20 minutes. After these 40 minutes, I introduced a second male (i.e., the intruder male) to the enclosure and recorded the behavior of the resident male for an additional 20 minutes. I attempted to size-match males, but not females, in trials (full range of male SVL = 20.3 mm; mean difference in male SVL in trials = 1.7 mm) and otherwise randomly created the salamander trios from animals collected at the same locality, selecting resident–intruder pairs irrespective of male morph. I used each male as a resident only once. To test my hypothesis, I was specifically interested in the behavior "bite", defined as a quick or extended bite motion from the resident male to the intruder male [36]. For all analyses, I excluded trials with females that were not obviously gravid. I compared the probability of "bite" as a function of resident male morph using a logistic regression and evaluated statistical significance using a likelihood-ratio test in `R` v3.4.2 [144].

4.2.3 Karyological analysis

To test whether this morphological polymorphism is related to the chromosomal inversion polymorphism, I karyotyped a subset of individuals from our behavioral trials. I selected a total of 8 adult male *E.* cf. *wilderae*—4 Morph A and 4 searching males—from a total of three disjunct populations representing both Lineages M and J [Figure 13]. Although this sample size would be too small to make a convincing case for perfect correspondence between morphology and karyotype, it is enough to reject the null hypothesis of no association if the morphs were karyologically distinct, and just a single counterexample would be adequate to

reject perfect correspondence between karyotype and morphology. I sent these salamanders to Hartwick College, where they were injected with a 0.1% solution of colchicine dissolved in phosphate-buffered saline (60% PBS). After approximately 24 hours, they were euthanized in a buffered MS-222 solution, dissected, and split, and intestines were cleaned. These tissues were then placed in distilled water for 10 minutes before being blotted and fixed with a 3:1 solution of ethanol:acetic acid. Chromosome spreads were then prepared, and each individual's karaymorph was diagnosed for the presence of the telocentric chromosomal inversion following Sessions [156].

4.2.4 Preliminary field observations

To test whether phenological differences observed by Bruce [19] were replicable, I collected two datasets. First, between October 2017 and January 2018, I opportunistically sampled for *Eurycea* cf. *wilderae* along the Little Pigeon River and its tributaries in Great Smoky Mountains National Park from approximately 35.7380°, -83.416° to 35.693°, -83.392°, which is within the distribution of Lineage M. I classified all observed individuals as either Morph A males, searching males, gravid females, or juveniles/non-reproductive adults by the presence of external secondary sexual characters, and I recorded whether I observed them on the forest floor (including the stream margin) or in the stream channel. These surveys consisted primarily of searching for surface-active salamanders on the forest floor and under stones in streams on rainy nights and searching under stones in streams during the day. I compared the relative distribution of Morph A males, searching males, gravid females, and juveniles/non-reproductive adults among habitats using Fisher's exact tests ($\alpha = 0.05$) in `R` v3.4.2 [144].

4.2.5 Additional field observations

Next, between 16 October 2018 and 11 November 2018, I conducted field surveys at Mill Creek within and adjacent to Highlands Biological Station in Macon County, North Carolina. This stream is dammed to form Lindenwood Lake, and I surveyed both above and below the lake. There, I conducted three types of surveys: 1) funnel traps placed along drift fences in terrestrial habitat; 2) nocturnal surveys of the forest floor; and 3) diurnal surveys

within the stream channel. I constructed one 30 m and two 20 m linear drift fences with silt fencing and placed a total of 20 window-screen funnel traps along them. Each funnel trap was approximately 13 x 13 x 38 cm in size, and I assembled them following the methods of Enge [49]. Because flooding, freezing temperatures, and other extenuating circumstances sometimes prevented the deployment of traps, I trapped for a total of 384 trap-nights. In the beginning of the sampling period, I surveyed for *Eurycea* active on the forest floor (i.e., I did not also search cover objects) for approximately 1–2 hours after sunset each night. Because this was unproductive on dry nights, I thereafter surveyed only on select rainy nights. Finally, I surveyed twice for salamanders in the stream channel by holding a dipnet flush with the substrate and disturbing leaf packs, stones, and gravel upstream (sensu Bruce [19]). I first conducted these surveys spread across five days in the beginning of the sampling period (17–24 October) and then again during the final day of sampling (11 November). During all surveys, I placed salamanders in individual, single-use plastic bags as I captured them. I recorded all data at the end of each survey and subsequently released salamanders at the point of capture. Thus, recaptures were not possible within a survey but were possible among surveys.

Based on secondary sexual characters, I assigned every metamorphosed *Eurycea* cf. *wilderae* to one of four forms: juveniles or non-reproductive adults, gravid females, searching males, or Morph A males. I used Neiko digital calipers to measure the snout-vent length (SVL) and head-width (HW) of 57 gravid females, 66 searching males, and 49 Morph A males. I visually examined all individuals for scars indicative of male-male combat and examined all females for sperm caps in their cloacas, which remain visible for up to 14 hours after insemination [147].

To compare SVL and HW/SVL ratios among forms, sampling periods, and methods, I used t-tests ($\alpha = 0.05$), and I plotted these data using SinaPlots using the package `sinaplot` in `R` v3.5.1 [168, 144]. Because sampling effort was uneven across survey types and sampling periods, I used Fisher's exact tests ($\alpha = 0.05$) to compare the relative frequencies of forms (i.e., rather than comparing absolute numbers) among survey types. I conducted all statistical analyses in `R` v3.5.1 [144].

4.3 Results

4.3.1 Phylogenetic analysis

After quality-filtering, I recovered between 486,156–3,253,139 (median = 987,728) paired-end reads per sample. I generated a final dataset of 23,891 loci (222,965 SNPs) found in at least five individuals, creating a data matrix with approximately 46% missing data for use in the phylogenetic analysis.

I recovered a well-supported phylogeny qualitatively similar to that inferred from the larger dataset from which these data were derived [Figure 14; Chapter 2]. Considering only the species with invariant male secondary sexual characters (i.e., not those with the putative polymorphism under examination here), this phylogeny suggests the monophyly of species with the guarding tactic (i.e., *Eurycea aquatica, E. junaluska, E. bislineata*, and *E.* cf. *cirrigera*), which are then sister to a clade with searching tactic (i.e., *E. cirrigera*). Similar to those from other studies [Chapter 2; 91, 106], this phylogeny suggests the paraphyly of both *E. cirrigera* and *E. wilderae*, and it confirms the presence of both Morph A and searching males in Lineage E. Most important, I recovered a sister relationship for each pair of Morph A and searching males collected from the same population, contrary to expectations if the two represented distinct cryptic, widely sympatric species. Thus, this phylogeny demonstrates that: 1) *Eurycea wilderae* sensu lato is paraphyletic; and 2) Morph A does not represent a cryptic, widely-sympatric species, but instead is an intraspecific polymorphism found in three putative species.

4.3.2 Behavioral trials

I observed biting in 5/32 trials with Morph A resident males (total = 9 bites) and 0/30 trials with searching male residents (total = 0 bites), indicating a significant difference in the probability of biting (df = 1; p = 0.008) [Figure 15]. All trials including biting occurred with animals from Lineage M. In one of these trials, a Morph A resident male persistently pursued and bit the intruder male, similar to behavior documented more commonly in Deitloff et al.

[36]. These results demonstrate that, at least in Lineage M, Morph A exhibits the guarding reproductive tactic found in monomorphic species.

4.3.3 Karyological analysis

All individuals—including both Lineages M and J and both morphs—were homozygous for the telocentric inversion (T/T) on chromosome 13 [Figure 16; Table 4]. This result rejects the hypothesis that the chromosome rearrangement is causally linked to the morphological polymorphism.

4.3.4 Preliminary field observations

During seven brief surveys between October 2017 and January 2018, I recorded 142 *E.* cf. *wilderae*, including 7 Morph A males, 38 searching males, 46 gravid females, and 51 juveniles/non-reproductive adults [Table 5]. Notably, I found no searching males or juveniles/non-reproductive adults in the stream until the final sampling period in January. The distribution of Morph A males was significantly different than those of searching males, females, and juveniles/non-reproductive adults ($p < 0.05$ in each case), but no significant difference exists among searching males, female, or juveniles/non-reproductive adults ($p > 0.05$ in each case).

4.3.5 Additional field observations

During surveys at Highlands Biological Station, I recorded a total of 18 captures of *Eurycea* cf. *wilderae* in funnel traps, 186 captures in nocturnal forest floor surveys, and 122 captures in diurnal stream surveys [Table 6; Table 7; Table 8]. In funnel trap surveys, I recorded a total of 2 captures of juveniles, 5 captures of females, 8 captures of searching males, and 3 captures of Morph A males. In forest floor surveys, I recorded a total of 90 captures of juveniles or non-reproductive adults, 25 captures of females, 71 captures of searching males, and 0 captures of Morph A males. In stream surveys, I recorded a total of 26 captures of juveniles, 47 captures of females, 3 captures of searching males, and 46 captures of Morph A males. Thus, 100% of captures of adult males during forest floor surveys were of searching

males, and 94% of captures of adult males during stream surveys were of Morph A males [Figure 17]. The relative frequencies of all pairwise combinations of forms (e.g., searching vs. Morph A) were significantly different between nocturnal forest floor surveys and diurnal stream surveys ($p < 0.05$ in all cases). Notably, the relative distribution of searching and guarding males also differed between nocturnal forest floor surveys and funnel trapping surveys ($p < 0.05$).

Across all surveys, females had larger SVL (mean = 39.3 mm, sd = 2.3) than Morph A males (mean = 38.3 mm, sd = 2.8), which in turn had larger SVL than searching males (mean = 36.9 mm, sd = 2.1), and all pairwise differences were significant (t = 5.92, 2.02, -2.80; $p < 0.05$); [Figure 18]. Guarding males had larger HW/SVL ratios (mean = 0.15, sd = 0.01) than females (mean = 0.13, sd = 0.01) or searching males (mean = 0.12, sd = 0.01), and again, all pairwise differences were significant (t = -12.04, 3.37, -16.59; $p < 0.05$) [Figure 18]. The mean SVL of Morph A males found during stream surveys in the beginning of the sampling period (38.7 mm, sd = 2.6) was larger than that from the end of the sampling period (37.9 mm, sd = 2.9 mm), although this difference was not significant (t = -0.88; $p < 0.39$). Females found in nocturnal forest floor surveys and diurnal stream surveys did not differ in SVL (t = 0.70; $p < 0.49$).

4.4 Discussion

Taken together, these data demonstrate that sympatric Morph A and searching males: 1) comprise an intraspecific morphological polymorphism; 2) have reproductive behavioral differences mirroring those found more broadly among *Eurycea* species with fixed guarding or searching male tactics; 3) do not appear to be related to the chromosomal inversion polymorphism; and 4) have divergent reproductive phenologies consistent with the reproductive niches of guarding and searching tactics. Thus, I conclude that these two male morphs represent alternative reproductive tactics. Because I have complete data from all aspects of this project (i.e., genomic, behavioral, karyological, and field observational) for only Lineage M, I can make the strongest argument for the existence of ARTs in this putative species. However, I documented the morphological polymorphism underlying these ARTs in Lineages

E, M, and J, and additional corroborating evidence supports the claim that these same ARTs exist in all three putative species.

I observed biting less frequently than a previous study of *Eurycea aquatica* using similar methods [36]. In this earlier study, resident males were allowed to acclimate to their containers for three days before females were added, and it is possible that the short acclimation period I used (20 minutes) contributed to the relative infrequency of observed biting. Future work increasing the acclimation time and time held with a female may improve the biological realism of observations. Regardless, I observed biting exclusively from Morph A males, similar to the observation of biting exclusively from the guarding male species in Deitloff et al. [36]. Anecdotal support for the frequency of biting comes from the observation of scars left by these bites on animals captured during and immediately following the breeding season. I have often observed these scars on the species with only guarding males, but rarely on species with only searching males; similarly, I have observed these scars commonly on Morph A males in Lineages E, M, and J, but rarely on searching males. Although I directly observed biting behavior from only Lineage M in our trials, I suspect that a lack of observations in Lineage J was due to a smaller sample size, and the presence of scars in all three putative species provides corroborating support for the existence of these ARTs in each one.

My preliminary field observational data from Lineage M complement those of Sever [160] and Bruce [19] and suggest phenological differences between alternative reproductive tactics in *Eurycea*. All of these data are also consistent with my anecdotal observations (of Lineages M and J) that when both male morphs are present in the vicinity of the stream, I tend to find Morph A males under rocks in the stream channel (i.e., underwater) and searching males in leaf litter or under rocks adjacent to the stream. My subsequent field observational data further corroborate these conclusions, demonstrating that male *Eurycea* with alternative reproductive tactics differ in reproductive phenology, with searching males remaining longer in terrestrial habitats while Morph A males are already present at aquatic nesting sites. I observed no Morph A males on nocturnal forest floor surveys, and I observed no searching males in diurnal stream surveys until the final day of the survey period. Thus, during this portion of the breeding season, males with alternative reproductive tactics are maximally

divergent in their positions on the landscape. The end of this period is likely marked by the increased frequency of subfreezing temperatures and end of suitable conditions for terrestrial courtship and feeding opportunities, leading to the arrival of searching males and juveniles to the stream to overwinter.

I observed tail-straddling walk between one searching male and one female on the forest floor, and I observed sperm caps in the cloacas of five additional females on forest floor surveys and two females in funnel traps. This confirms that, in addition to other explanations for activity on the forest floor (e.g., feeding or migration), searching males are actively courting females during this period. During a stream survey on the final day of sampling, I observed a single female with a sperm cap in her cloaca. During this same survey, I observed fresh wounds suggestive of male-male aggression documented in mate-guarding trials [36] on two Morph A males. This evidence alone is insufficient to conclude that courtship is delayed in Morph A males, but it is consistent with results from earlier research that support this hypothesis. The greater proclivity of Morph A males to court in the laboratory late in the reproductive season led [147] to hypothesize that courtship occurs earlier for searching males than for Morph A males. Furthermore, she documented two pairs of Morph A males and females with sperm caps in their cloacas under rocks in a stream, suggesting that courtship likely occurs in aquatic habitats for Morph A males. In total, these data support the conclusion that searching males first court females in terrestrial habitats, and Morph A males court them later in aquatic habitats. Thus, these field observational data reveal the nature of the divergent reproductive niches underlying these ARTs, with searching males suited for locating and courting females in terrestrial habitats and guarding males suited for mate-guarding near nests in aquatic habitats.

The difference in relative frequency of searching and Morph A males in funnel traps vs. forest floor surveys suggests a bias in sampling, as both methods are designed to sample the same (i.e., terrestrial) portion of the population. One likely explanation is a difference in detection probability due to behavior, as the majority of animals sampled on nocturnal forest floor surveys were climbing on aboveground vegetation. If some Morph A males remain on the forest floor but do not actively climb in search of courtship or feeding opportunities, or if they remain underground or under cover objects, this survey method could be biased

against them. Additionally, Morph A males are typically duller in color than searching males, potentially magnifying this difference in detection probability. A careful capture-mark-recapture study would provide a quantitative answer to this question. It may be notable that Morph A males found during diurnal stream surveys at the end of the sampling period were smaller than those found in the beginning of the sampling period, although this difference was not significant. Two of the three Morph A males found in funnel traps were among the smallest individuals captured throughout this study, potentially suggesting the later arrival of smaller Morph A males.

The heritability and long-term flexibility of these ARTs remain unknown. Differences in mean SVL and mean HW/SVL ratios I observed among females, searching, and Morph A males are consistent with results from Bruce [19], who interpreted the difference in SVL between searching and Morph A males as potential evidence of an ontogenetic change among morphologies. While this is still a possibility, there is no direct evidence for this hypothesis. At the very least, these strategies and their associated discrete morphological traits appear to be fixed within a reproductive season, but I strongly suspect that they persist longer. Genealogical data, mark-recapture data, and long-term observation of captive individuals could provide more definitive answers to these questions of ART heritability and flexibility. The larger HW/SVL ratio of guarding males is consistent with the use of these seasonally hypertrophied jaw muscles in mate-guarding behavior [58, 36, 141]. Because nesting occurs exclusively in aquatic habitats, it is hypothesized that mate-guarding also occurs there, rather than in terrestrial habitats through which the salamanders migrate [58, 36].

Although I found no evidence for a clear causal relationship between the inversion polymorphism and these ARTs in Lineages M and J, the topic warrants further research. Chromosome 13 in *E.* cf. *wilderae* exists as either telocentric (T) or subtelocentric (ST). Since the inversion is only found in *E.* cf. *wilderae* and not in any other species of the genus that have been examined (*E. bislineata, E. cirrigera, E. junaluska, E. longicauda, and E. guttolineata*), the inversion leading to telocentric chromosome 13 is probably derived in these species [157, Sessions, unpublished data]. In some populations of *E.* cf. *wilderae*, the inversion exists as a floating polymorphism with all three karyomorphs (ST/ST, ST/T, and T/T) present. The majority of populations of *E.* cf. *wilderae*, however, are fixed for the

T/T karyomorph [157], including the specimens sampled here. Future research should more closely examine the prevalence and evolutionary history of this inversion in other putative species in the *E. bislineata* species complex.

Nussbaum [133, 132] summarizes research on evolution of parental care in salamanders and describes several prominent patterns: 1) biparental care is absent or very uncommon; and 2) all species with male parental care have external fertilization, but female parental care is relatively common in species with internal fertilization. While several competing hypotheses attempt to explain this pattern, Nussbaum [133] suggests that the sex that selects an oviposition site is the sex that become the caregiver. Because species with internal fertilization (e.g., all plethodontid salamanders) often separate courtship from oviposition by long periods of time, these species exhibit only female parental care. Male salamanders of some guarding species in the *E. bislineata* species complex—*Eurycea aquatica* [58], *E. junaluska* [17], *E.* cf. *aquatica* [142], *E.* cf. *cirrigera* [89]—have been found in attendance of eggs with or without females, but males of the searching species *Eurycea cirrigera* have not. I know of observations of Morph A males within the likely geographic distribution of both Lineage M [Camp, unpublished data] and Lineage J [Bennett, unpublished data] found in the presence of nests, but I have never observed searching males in similar situations. This again supports the existence of these ARTs in more than one putative species and suggests a link between mate-guarding and male parental care. Nussbaum [132] summarizes other cases of male plethodontids found in the presence of nests in species believed to have only female parental care and suggests that these cases likely represent attempted sexual interference or chance encounters. While this may also be the case for guarding male *Eurycea*, the possibility that these males select nesting sites and some observations of the same male found repeatedly with the same nest [58] suggest the possibility of true biparental care. Further careful observations of these behaviors in the field and in captivity will help answer these questions.

The evolutionary history of these reproductive strategies is still unclear. Within the *E. bislineata* species complex, excluding species with these ARTs, there exists one clade of guarding species and one clade of searching species. The three putative species with these ARTs (i.e., Lineages E, M, and J) are paraphyletic, but secondary contact and

introgression have occurred between them [Chapter 2]. It is therefore possible that these ARTs: 1) represent the ancestral state of the *E. bislineata* species complex; 2) have evolved independently at least twice; or 3) have moved between species through introgressive hybridization. The evolutionary instability of ARTs in other organisms [3] and the complex evolutionary history in this group prevent us from speculating further, but the investigation of variation among these putative species may provide answers. Sever [162] notes Morph A males from the Piedmont and Coastal Plain of the Carolinas, which have been traditionally described as *E. cirrigera*, but these belong to Lineage J [106]. However, it is possible that multiple reproductive tactics may one day be confirmed in other members of the *E. bislineata* species complex that are currently thought to be monomorphic (e.g., Lineage L). In some other species of spelerpine plethodontids (e.g., *Pseudotriton*; *Eurycea tynerensis*), males appear to have hypertrophied jaw musculature, but I know little about their reproductive behaviors, although Arnold [6] describes potential mate-guarding behavior in *Pseudotriton ruber*. Other distantly-related plethodontid salamanders (e.g., *Aneides*) have superficially similar jaw musculature that appears to be correlated with territorial behavioral (and not mate-guarding), but the homology of these morphological traits and behaviors is unknown [173, 35]. Further research is warranted into the conditions underlying the evolution and maintenance (or fixation) of ARTs in plethodontid salamanders.

Chapter 5

Conclusion

Describing the mechanisms structuring the distribution of genetic and phenotypic diversity is a central goal in ecology and evolutionary biology. These mechanisms operate across temporal and spatial scales—from continental geological change across eons to species recognition during a courtship event—to influence gene flow among and within species. Understanding these mechanisms is important for reconstructing phylogeographic histories and delimiting modern species boundaries. Here, I explored these themes in the two-lined salamander (*Eurycea bislineata*) species complex—a group of semiaquatic plethodontid salamanders from the eastern United States and Canada— and demonstrated: 1) a reticulate evolutionary history, with putative species boundaries coinciding with river drainage boundaries and historical hybridization and introgression in regions with dynamic histories of river drainage reorganization; 2) reproductive isolation and fine-scale ecological segregation between two species currently found in sympatry; 3) two morphologically distinct, alternative reproductive tactics in males. These results reveal the complex evolutionary history of this group of salamanders and contribute more broadly to our understanding of reticulate evolutionary histories, secondary contact, and reproductive diversity across taxa.

Bibliography

[1] Alcorn, M. A., Deitloff, J., Graham, S. P., and Timpe, E. K. (2013). Sexual dimorphism in head shape, relative head width, and body size of *Eurycea aquatica* and *Eurycea cirrigera*. *Journal of Herpetology*, 47(2):321–327. 44

[2] Alexandrino, J., Baird, S. J., Lawson, L., Macey, J. R., Moritz, C., and Wake, D. B. (2005). Strong selection against hybrids at a hybrid zone in the *Ensatina* ring species complex and its evolutinary implications. *Evolution*, 59(6):1334–1347. 4

[3] Almada, V. C. and Robalo, J. I. (2008). Phylogenetic analysis of alternative reproductive tactics: problems and possibilities. In Oliveira, R. F., Taborsky, M., and Brockmann, H. J., editors, *Alternative reproductive tactics, an integrative approach*, pages 52–62. Cambridge University Press. 7, 58

[4] AmphibiaWeb (2019). AmphibiaWeb. https://www.amphibiaweb.org. 1, 8

[5] Andrews Jr, W. M. (2004). *Geologic controls on Plio-Pleistocene drainage evolution of the Kentucky River in central Kentucky*. PhD thesis, University of Kentucky. 28, 32

[6] Arnold, S. J. (1972). *The evolution of courtship behavior in salamanders*. PhD thesis, University of Michigan. 45, 58

[7] Arnold, S. J. (1976). Sexual behavior, sexual interference and sexual defense in the salamanders *Ambystoma maculatum*, *Ambystoma tigrinum* and *Plethodon jordani*. *Ethology*, 42(3):247–300. 5, 7

[8] Avise, J. C. (2000). *Phylogeography: the history and formation of species*. Harvard University Press. 2

[9] Barton, N. H. and Hewitt, G. M. (1985). Analysis of hybrid zones. *Annual Review of Ecology and Systematics*, 16(1):113–148. 1, 4

[10] Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using `lme4`. *Journal of Statistical Software*, 67(1):1–48. 40

[11] Bayona-Vásquez, N. J., Glenn, T. C., Kieran, T. J., Pierson, T. W., Hoffberg, S. L., Scott, P. A., Bentley, K. E., Jr., J. W. F., Louha, S., Troendle, N., Diaz-Jaimes,

P., Mauricio, R., and Faircloth, B. C. (2019). Adapterama III: Quadruple-indexed, double/triple-enzyme RADseq libraries (2RAD/3RAD). *bioRxiv*. 15, 35, 36, 47, 109

[12] Beachy, C. K. (1996). Reduced courtship success between parapatric populations of the plethodontid salamander *Gyrinophilus porphyriticus*. *Copeia*, 1996(1):199–203. 6

[13] Beane, J. C., Braswell, A. L., Mitchell, J. C., and Palmer, W. M. (2010). *Amphibians and Reptiles of the Carolinas and Virginia*. University of North Carolina Press. 32

[14] Bishop, P. (1995). Drainage rearrangement by river capture, beheading and diversion. *Progress in Physical Geography*, 19(4):449–473. 3

[15] Bonett, R. M., Steffen, M. A., Lambert, S. M., Wiens, J. J., and Chippindale, P. T. (2014). Evolution of paedomorphosis in plethodontid salamanders: ecological correlates and re-evolution of metamorphosis. *Evolution*, 68(2):466–482. 11, 23

[16] Brown, W. L. and Wilson, E. O. (1956). Character displacement. *Systematic Zoology*, 5(2):49–64. 4

[17] Bruce, R. C. (1982). Egg-laying, larval periods and metamorphosis of *Eurycea bislineata* and *E. junaluska* at Santeetlah Creek, North Carolina. *Copeia*, 1982:755–762. 57

[18] Bruce, R. C. (1986). Upstream and downstream movements of *Eurycea bislineata* and other salamanders in a southern Appalachian stream. *Herpetologica*, pages 149–155. 10

[19] Bruce, R. C. (1988). An ecological life table for the salamander *Eurycea wilderae*. *Copeia*, 1988(1):15–26. 19, 46, 47, 49, 50, 54, 56

[20] Bryant, D. and Moulton, V. (2004). Neighbor-Net: an agglomerative method for the construction of phylogenetic networks. *Molecular Biology and Evolution*, 21(2):255–265. 17

[21] Buerkle, C. A. and Lexer, C. (2008). Admixture as the basis for genetic mapping. *Trends in Ecology & Evolution*, 23(12):686–694. 4

[22] Bulmer, M. (1972). Multiple niche polymorphism. *The American Naturalist*, 106(948):254–257. 7

[23] Burbrink, F. T. and Gehara, M. (2018). The biogeography of deep time phylogenetic reticulation. *Systematic Biology*, 67(5):743–744. 2, 31

[24] Burridge, C., Craw, D., and Waters, J. (2006). River capture, range expansion, and cladogenesis: the genetic signature of freshwater vicariance. *Evolution*, 60(5):1038–1049. 3

[25] Cadena, C. D. (2007). Testing the role of interspecific competition in the evolutionary origin of elevational zonation: an example with Buarremon brush-finches (Aves, Emberizidae) in the Neotropical mountains. *Evolution: International Journal of Organic Evolution*, 61(5):1120–1136. 4

[26] Camp, C., Peterman, W., Milanovich, J., Lamb, T., Maerz, J., and Wake, D. (2009). A new genus and species of lungless salamander (family Plethodontidae) from the Appalachian highlands of the south-eastern United States. *Journal of Zoology*, 279(1):86–94. 44

[27] Camp, C. D., Marshall, J. L., Landau, K. R., Austin Jr, R. M., and Tilley, S. G. (2000). Sympatric occurrence of two species of the two-lined salamander (*Eurycea bislineata*) complex. *Copeia*, 2000(2):572–578. 28, 33, 34, 35, 42

[28] Camp, C. D., Wooten, J. A., Corbet, C. M., Dulka, E. A., Mitchem, J. A., and Krieger, T. J. (2013). Ecological interactions between two broadly sympatric, cryptic species of dusky salamander (genus *Desmognathus*). *Copeia*, 2013(3):499–506. 5

[29] Campbell, M. R. (1894). Tertiary changes in the drainage of southwestern Virginia. *American Journal of Science*, 48(283):21. 27

[30] Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., and Cresko, W. A. (2013). `Stacks`: an analysis tool set for population genomics. *Molecular Ecology*, 22(11):3124–3140. 38

[31] Combellas-Bigott, R. I. and Galloway, W. E. (2006). Depositional and structural evolution of the middle Miocene depositional episode, east-central Gulf of Mexico. *AAPG Bulletin*, 90(3):335–362. 14

[32] Cooper, J. E. (2006). A new species of crayfish of the genus *Cambarus* Erichson, 1846 (Decapoda: Cambaridae) from the eastern Blue Ridge foothills and western Piedmont Plateau of North Carolina. *Proceedings of the Biological Society of Washington*, 119(1):67–81. 13

[33] Coyne, J. A. and Orr, H. A. (1989). Patterns of speciation in *Drosophila*. *Evolution*, 43(2):362–381. 5

[34] Coyne, J. A. and Orr, H. A. (2004). *Speciation.* Sinauer Associates, Inc. 2

[35] Cupp, Jr., P. V. (1980). Territoriality in the green salamander, *Aneides aeneus*. *Copeia*, 1980:463–468. 58

[36] Deitloff, J., Alcorn, M. A., and Graham, S. P. (2014). Variation in mating systems of salamanders: mate guarding or territoriality? *Behavioural Processes*, 106:111–117. 44, 45, 48, 52, 54, 55, 56

[37] Devitt, T. J., Baird, S. J., and Moritz, C. (2011). Asymmetric reproductive isolation between terminal forms of the salamander ring species *Ensatina eschscholtzii* revealed by fine-scale genetic analysis of a hybrid zone. *BMC Evolutionary Biology*, 11(1):245. 4

[38] Devitt, T. J., Wright, A. M., Cannatella, D. C., and Hillis, D. M. (2019). Species delimitation in endangered groundwater salamanders: Implications for aquifer management and biodiversity conservation. *Proceedings of the National Academy of Sciences*, 116:2624–2633. 2

[39] Diamond, J. M. (1973). Distributional ecology of New Guinea birds: recent ecological and biogeographical theories can be tested on the bird communities of New Guinea. *Science*, 179(4075):759–769. 4

[40] Dietrich, R. V. (1959). *Geology and mineral resources of Floyd County of the Blue Ridge Upland, southwestern Virginia*. Number 134 in Bulletin of the Engineering Experimental Station Series. Virginia Polytechnic Institute and State University. 13

[41] Dray, S. and Dufour, A.-B. (2007). The `ade4` package: implementing the duality diagram for ecologists. *Journal of Statistical Software*, 22(4):1–20. 17

[42] Dufour, L. (1844). Anatomie générale des Dipteres. *Annales des Sciences Naturelles*, 1:244–264. 5

[43] Dunn, E. R. (1920). Some reptiles and amphibians from Virginia, North Carolina, Tennessee and Alabama. *Proceedings of the Biological Society of Washington*, 33:129–137. 8, 44

[44] Dunn, E. R. (1926). *The salamanders of the family Plethodontidae*. Smith College. 8

[45] Dunn, P. O., Whittingham, L. A., and Pitcher, T. E. (2001). Mating systems, sperm competition, and the evolution of sexual dimorphism in birds. *Evolution*, 55(1):161–175. 1

[46] Eaton, D. and Overcast, I. (2017). `ipyrad`: Interactive assemby and analysis of RAD-seq data sets. https://github.com/dereneaton/ipyrad. 16, 36, 38, 47

[47] Eaton, D. A. (2014). `PyRAD`: assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics*, 30(13):1844–1849. 16, 18, 36, 38, 47

[48] Eaton, D. A. and Ree, R. H. (2013). Inferring phylogeny and introgression using RADseq data: an example from flowering plants (Pedicularis: Orobanchaceae). *Systematic Biology*, 62(5):689–706. 14, 16

[49] Enge, K. M. (1998). Herpetofaunal drift-fence survey of steephead ravines in the Apalachicola and Ochlockonee River drainages. *Florida Game and Fresh Water Fish Commission*. 50

[50] Engqvist, L. and Taborsky, M. (2016). The evolution of genetic and conditional alternative reproductive tactics. *Proceedings of the Royal Society of London B: Biological Sciences*, 283(1825):20152945. 6

[51] Etnier, D. A. and Starnes, W. C. (1993). *The Fishes of Tennessee*. University of Tennessee Press. 14

[52] Excoffier, L., Dupanloup, I., Huerta-Sánchez, E., Sousa, V. C., and Foll, M. (2013). Robust demographic inference from genomic and SNP data. *PLoS genetics*, 9(10):e1003905. 18

[53] Fitzpatrck, B., Placyk Jr., J., Niemiller, M. L., Casper, G. S., and Burghardt, G. M. (2008). Distinctiveness in the face of gene flow: hybridization between specialist and generalist gartersnakes. *Molecular Ecology*, 17(18):4107–4117. 38

[54] Gallen, S. F. (2018). Lithologic controls on landscape dynamics and aquatic species evolution in post-orogenic mountains. *Earth and Planetary Science Letters*, 493:150–160. 14, 29

[55] Ghitea, O. and Sattler, P. (1990). The distribution and identification of two-lined salamanders in Virginia. *Catesbeiana*, 10:11–18. 32

[56] Goodman, S. N. (1999). Toward evidence-based medical statistics. 2: The Bayes factor. *Annals of Internal Medicine*, 130(12):1005–1013. 39

[57] Goudet, J. (2005). `hierfstat`, a package for `R` to compute and test hierarchical F-statistics. *Molecular Ecology Notes*, 5(1):184–186. 39

[58] Graham, S., Timpe, E., Hoss, S., Alcorn, M., and Deitloff, J. (2010). Notes on reproduction in the brownback salamander (*Eurycea aquatica*). *IRCF Reptiles and Amphibians*, 17:168–172. 56, 57

[59] Graham, S. P., Alcorn, M. A., Timpe, E. K., and Deitloff, J. (2013). Seasonal changes of primary and secondary sexual characteristics in the salamanders *Eurycea aquatica* and *E. cirrigera*. *Herpetological Conservation and Biology*, 8(1):53–64. 44

[60] Green, J. (1818). Descriptions of several species of North American Amphibia, accompanied with observations. *Journal of Academy of Natural Science, Philadelphia*, 1:348–358. 8

[61] Green, J. (1831). Description of two new species of salamander. *Journal of Academy of Natural Science, Philadelphia*, 6(2):253–255. 8

[62] Green, R. E., Krause, J., Briggs, A. W., Maricic, T., Stenzel, U., Kircher, M., Patterson, N., Li, H., Zhai, W., Fritz, M. H.-Y., et al. (2010). A draft sequence of the Neandertal genome. *Science*, 328(5979):710–722. 1

[63] Gross, M. R. (1996). Alternative reproductive strategies and tactics: diversity within sexes. *Trends in Ecology & Evolution*, 11(2):92–98. 6

[64] Gross, M. R. and Charnov, E. L. (1980). Alternative male life histories in bluegill sunfish. *Proceedings of the National Academy of Sciences*, 77(11):6937–6940. 6

[65] Guttman, S. I. and Karlin, A. A. (1986). Hybridization of cryptic species of two-lined salamanders (*Eurycea bislineata* complex). *Copeia*, 1986:96–108. 32, 43

[66] Haldane, J. (1948). The theory of a cline. *Journal of Genetics*, 48(3):277–284. 4

[67] Hardin, G. (1960). The competitive exclusion principle. *Science*, 131(3409):1292–1297. 4

[68] Harrison, R. G. (1986). Pattern and process in a narrow hybrid zone. *Heredity*, 56(3):337. 4

[69] Harrison, R. G. (1990). Hybrid zones: windows on evolutionary process. *Oxford Surveys in Evolutionary Biology*, 7:69–128. 3

[70] Harrison, R. G. and Larson, E. L. (2016). Heterogeneous genome divergence, differential introgression, and the origin and structure of hybrid zones. *Molecular Ecology*, 25(11):2454–2466. 4

[71] Harrison, R. G. and Rand, D. M. (1989). Mosaic hybrid zone and the nature of species boundaries. In Otte, D. and Endler, J. A., editors, *Speciation and its Consequences*. Sinauer Associates. 4

[72] Haughey, M. D. (2015). *Phylogeography of the spring salamander,* Gyrinophilus porphyriticus*: Historic and Contemporary River System's Influence on Phylogeographic History*. PhD thesis, Ohio University. 13

[73] Hayes, C. W. and Campbell, M. R. (1894). *Geomorphology of the southern Appalachians*. National Geographic Society. 14

[74] Hazel, W., Smock, R., and Lively, C. M. (2004). The ecological genetics of conditional strategies. *The American Naturalist*, 163(6):888–900. 7

[75] Hewitt, G. M. (1988). Hybrid zones—natural laboratories for evolutionary studies. *Trends in Ecology & Evolution*, 3(7):158–167. 3

[76] Hewitt, G. M. (2001). Speciation, hybrid zones and phylogeography—or seeing genes in space and time. *Molecular Ecology*, 10(3):537–549. 1, 2, 31

[77] Highton, R., Maha, G. C., and Maxson, L. R. (1989). *Biochemical evolution in the slimy salamanders of the* Plethodon glutinosus *complex in the Eastern United States*. University of Illinois Press. 1, 2

[78] Highton, R. and Peabody, R. B. (2000). Geographic protein variation and speciation in salamanders of the *Plethodon jordani* and *Plethodon glutinosus* complexes in the southern Appalachian Mountains with the description of four new species. In *The Biology of Plethodontid Salamanders*, pages 31–93. Springer. 2, 4

[79] Hobbs Jr, H. H. (1969). On the distribution and phylogeny of the crayfish genus *Cambarus*. In Holt, P. C., Parker, B. C., and Roane, M. K., editors, *The distributional history of the biota of the southern Appalachians*, volume I: Invertebrates, pages 93–178. Virginia Polytechnic Institute and State University. 27

[80] Hocutt, C., Jenkins, R., and Stauffer Jr, J. (1986). Zoogeography of the fishes of the central Appalachians and central Atlantic coastal plain. In Hocutt, C. and Wiley, E., editors, *The Zoogeography of North American Freshwater Fishes*, pages 161–211. Wiley. 13, 24

[81] Hocutt, C. H. (1979). Drainage evolution and fish dispersal in the central Appalachians. *Geological Society of America Bulletin*, 90:197–234. 13, 24

[82] Hocutt, C. H. and Wiley, E. O. (1986). *The Zoogeography of North American Freshwater Fishes*. Wiley. 2, 3

[83] Hoffberg, S. L., Kieran, T. J., Catchen, J. M., Devault, A., Faircloth, B. C., Mauricio, R., and Glenn, T. C. (2016). RADcap: sequence capture of dual-digest RADseq libraries with identifiable duplicates and reduced missing data. *Molecular Ecology Resources*, 16(5):1264–1278. 35, 36

[84] Houck, L. D. (1988). The effect of body size on male courtship success in a plethodontid salamander. *Animal Behaviour*, 36(3):837–842. 6

[85] Houck, L. D. and Arnold, S. J. (2003). Courtship and mating behavior. *Reproductive biology and phylogeny of Urodela*, 1:383–424. 5

[86] Houck, L. D., Arnold, S. J., and Hickman, A. R. (1988). Tests for sexual isolation in plethodontid salamanders (Genus *Desmognathus*). *Journal of Herpetology*, pages 186–191. 6, 44

[87] Houck, L. D. and Verrell, P. A. (1993). Studies of courtship behavior in plethodontid salamanders: a review. *Herpetologica*, 49(2):175–184. 5

[88] Huson, D. H., Klöpper, T., Lockhart, P. J., and Steel, M. A. (2005). Reconstruction of reticulate networks from gene trees. In *Annual International Conference on Research in Computational Molecular Biology*, pages 233–249. Springer. 18

[89] Hutton, J. M. and Pierson, T. W. (2017). *Eurycea bislineata* (Northern Two-lined Salamander) Nesting. *Herpetological Review*, 48(4):826. 57

[90] Irwin, D. E. and Price, T. (1999). Sexual imprinting, learning and speciation. *Heredity*, 82(4):347. 1

[91] Jacobs, J. F. (1987). A preliminary investigation of geographic variation and systematics of the two-lined salamander, *Eurycea bislineata* (Green). *Herpetologica*, 43(4):423–446. 8, 11, 12, 15, 23, 44, 45, 51

[92] Jaeger, R. G. (1971). Competitive exclusion as a factor influencing the distributions of two species of terrestrial salamanders. *Ecology*, 52(4):632–637. 5

[93] Janssen, R. E. (1952). The history of a river. *Scientific American*, 186(6):74–82. 13

[94] Jenkins, R. E., Schwartz, F. J., and Lachner, E. A. (1971). Fishes of the central Appalachian drainages: their distribution and dispersal. In Holt, P. C., Parker, B. C., and Roane, M. K., editors, *The distributional history of the biota of the southern Appalachians*, volume III: Vertebrates, pages 43–117. Virginia Polytechnic Institute and State University. 27

[95] Jockusch, E. L. and Wake, D. B. (2002). Falling apart and merging: diversification of slender salamanders (Plethodontidae: *Batrachoseps*) in the American West. *Biological Journal of the Linnean Society*, 76(3):361–391. 1, 2, 3, 5

[96] Johnson, D. W. (1905). The Tertiary history of the Tennessee River. *The Journal of Geology*, 13(3):194–231. 14

[97] Johnson, J. E. and Goldberg, A. S. (1975). Movement of larval two lined salamanders (*Eurycea bislineata*) in the Mill River, Massachusetts. *Copeia*, 1975(3):588–589. 10

[98] Jombart, T. and Ahmed, I. (2011). `adegenet` 1.3-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics*, 27(21):3070–3071. 39

[99] Jones, M. T., Voss, S. R., Ptacek, M. B., Weisrock, D. W., and Tonkyn, D. W. (2006). River drainages and phylogeography: an evolutionary significant lineage of shovel-nosed salamander (*Desmognathus marmoratus*) in the southern Appalachians. *Molecular Phylogenetics and Evolution*, 38(1):280–287. 2

[100] Jones, T. R. (1980). A reevaluation of the salamander, *Eurycea aquatica* Rose and Bush (Amphibia: Plethodontidae). Master's thesis, Auburn University. 8, 32

[101] Judson, S. (1975). Evolution of Appalachian topography. *Publications in Geomorphology*, Theories of Landform Development:29–44. 27

[102] Kass, R. E. and Raftery, A. E. (1995). Bayes factors. *Journal of the American Statistical Association*, 90(430):773–795. 39

[103] Kawamichi, T. and Ueda, H. (1998). Spawning at nests of extra-large males in the giant salamander *Andrias japonicus*. *Journal of Herpetology*, 32(1):133–136. 7

[104] Keith, A. (1925). The Highlands. In La Forge, L., Cooke, W., Keith, A., and Campbell, M., editors, *Physical Geography of Georgia*, pages 93–132. Geological Survey Georgia. 28

[105] Kozak, K. H. (2003). Sexual isolation and courtship behavior in salamanders of the *Eurycea bislineata* species complex, with comments on the evolution of the mental gland and pheromone delivery behavior in the Plethodontidae. *Southeastern Naturalist*, 2(2):281–292. 33, 43, 45

[106] Kozak, K. H., Blaine, R. A., and Larson, A. (2006). Gene lineages and eastern North American palaeodrainage basins: phylogeography and speciation in salamanders of the *Eurycea bislineata* species complex. *Molecular Ecology*, 15:191–207. 2, 8, 11, 12, 13, 14, 15, 20, 22, 23, 24, 25, 26, 27, 28, 32, 34, 42, 44, 45, 46, 51, 58, 84, 85, 94, 141

[107] Kozak, K. H. and Montanucci, R. R. (2001). Genetic variation across a contact zone between montane and lowland forms of the two-lined salamander (*Eurycea bislineata*) species complex: a test of species limits. *Copeia*, 2001(1):25–34. 28, 33

[108] Kozak, K. H. and Wiens, J. (2006). Does niche conservatism promote speciation? A case study in North American salamanders. *Evolution*, 60(12):2604–2621. 5

[109] Kozak, K. H. and Wiens, J. J. (2010). Niche conservatism drives elevational diversity patterns in Appalachian salamanders. *The American Naturalist*, 176(1):40–54. 5

[110] Kozlov, A., Darriba, D., Flouri, T., Morel, B., and Stamatakis, A. (2018). `RAxML-NG`: A fast, scalable, and user-friendly tool for maximum likelihood phylogenetic inference. *bioRxiv*. 16

[111] Kuchta, S. R., Haughey, M., Wynn, A. H., Jacobs, J. F., and Highton, R. (2016). Ancient river systems and phylogeographical structure in the spring salamander, *Gyrinophilus porphyriticus*. *Journal of Biogeography*, 43(4):639–652. 2

[112] Küpper, C., Stocks, M., Risse, J. E., dos Remedios, N., Farrell, L. L., McRae, S. B., Morgan, T. C., Karlionova, N., Pinchuk, P., Verkuil, Y. I., Kitaysky, A. S., Wingfield, J. C., Piersma, T., Zeng, K., Slate, J., Blaxter, M., Lank, D. B., and Burke, T. (2016). A supergene determines highly divergent male reproductive morphs in the ruff. *Nature Genetics*, 48(1):79. 47

[113] Kurdziel, J. P. and Knowles, L. L. (2002). The mechanisms of morph determination in the amphipod *Jassa*: implications for the evolution of alternative male phenotypes. *Proceedings of the Royal Society of London B: Biological Sciences*, 269(1502):1749–1754. 7

[114] Langellotto, G. A. and Denno, R. F. (2001). Benefits of dispersal in patchy environments: mate location by males of a wing-dimorphic insect. *Ecology*, 82(7):1870–1878. 6

[115] Langellotto, G. A., Denno, R. F., and Ott, J. R. (2000). A trade-off between flight capability and reproduction in males of a wing-dimorphic insect. *Ecology*, 81(3):865–875. 6

[116] Leaché, A. D., Harris, R. B., Rannala, B., and Yang, Z. (2013). The influence of gene flow on species tree estimation: a simulation study. *Systematic Biology*, 63(1):17–30. 14

[117] Linck, E. and Battey, C. (2019). Minor allele frequency thresholds strongly affect population structure inference with genomic datasets. *Molecular Ecology Resources*. 38, 39

[118] Lowe, W. H., Mcpeek, M. A., Likens, G. E., and Cosentino, B. J. (2008). Linking movement behaviour to dispersal and divergence in plethodontid salamanders. *Molecular Ecology*, 17(20):4459–4469. 10

[119] MacArthur, R. H. and Wilson, E. O. (1967). *The Theory of Island Biogeography*. Princeton University Press. 5

[120] MacCulloch, R. D. and Bider, J. R. (1975). Phenology, migrations, circadian rhythm and the effect of precipitation of the activity of *Eurycea b. bislineata* in Quebec. *Herpetologica*, pages 433–439. 10

[121] Marshall, J. L. (2000). *Alternative mechanisms of character divergence: theoretical and empirical consideration*. PhD thesis, University of Louisiana at Lafayette. 33, 34, 35, 42

[122] Mayden, R. L. and Matson, R. H. (1992). Systematics and biogeography of the Tennessee shiner, *Notropis leuciodus* (Cope) (Teleostei: Cyprinidae). *Copeia*, 1992:954–968. 3

[123] Mayr, E. (1942). *Systematics and the origin of species, from the viewpoint of a zoologist*. Columbia University Press. 2

[124] Mayr, E. (1947). Ecological factors in speciation. *Evolution*, 1(4):263–288. 5, 44

[125] Melhorn, W. N. and Kempton, J. P. (1991). *Geology and Hydrogeology of the Teays-Mahomet Bedrock Valley System*. Geological Society of America. 28, 32

[126] Miller, D. and Hallerman, E. (1994). Isozyme survey of two-lined salamander (*Eurycea bislineata*) populations in Maryland and Virginia. *Bulletin of the Maryland Herpetological Society*, 30:78–97. 33, 43

[127] Mittleman, M. B. (1949). American Caudata. VI. The races of *Eurycea bislineata*. *Proceedings of The Biological Society of Washington*, 62:89–96. 8

[128] Mittleman, M. B. (1967). *Eurycea bislineata*. *Catalogue of American Amphibians and Reptiles*. 8

[129] Moritz, C., Schneider, C. J., and Wake, D. B. (1992). Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Systematic Biology*, 41(3):273–291. 2

[130] Mount, R. (1980). *The Reptiles and Amphibians of Alabama*. University Alabama Press. 8

[131] Nagle, B. C. (2010). Early Quaternary sea level changes and rapid diversification in the North American minnow genus *Nocomis* (Ostariophysi: Cyprinidae). Master's thesis, University of Minnesota. 13

[132] Nussbaum, R. (2003). Parental care. In Sever, D. M., editor, *Reproductive biology and phylogeny of Urodela*, pages 527–612. Science Publishers, Inc. 57

[133] Nussbaum, R. A. (1985). The evolution of parental care in salamanders. *Miscellaneous Publications of the Museum of Zoology, University of Michigan*, 169:1–50. 57

[134] Oliveira, R. F., Taborsky, M., and Brockmann, H. J., editors (2008). *Alternative reproductive tactics: an integrative approach*. Cambridge University Press. 6

[135] Oliver, J. A. and Bailey, J. R. (1939). Amphibians and reptiles of New Hampshire. *New Hampshire Fish and Game Department*, 4:195–221. 8

[136] Overcast, I. (2017). `easySFS`. https://www.github.com/isaacovercast/easySFS. 19

[137] Persons, A. K. (2010). *Geological and ichthyological investigations into palaeodrainage hypothesis for the Tennessee River*. PhD thesis, University of Southern Mississippi. 3, 14

[138] Petranka, J. W. (1998). *Salamanders of the United States and Canada*. Smithsonian Institution Press. 1, 2, 10, 12, 32, 33, 43

[139] Philipp, D. and Gross, M. (1994). Genetic evidence for cuckoldry in bluegill *Lepomis macrochirus*. *Molecular Ecology*, 3(6):563–569. 6

[140] Pierson, T. (In Press). Divergent reproductive phenologies in male *Eurycea* with alternative reproductive tactics. *Herpetological Review*. iv, 44

[141] Pierson, T. W., Deitloff, J., Sessions, S. K., Kozak, K. H., and Fitzpatrick, B. M. (2019). Morphological polymorphism associated with alternative reproductive tactics in a plethodontid salamander. *The American Naturalist*, 193(4). iv, 44, 56

[142] Pierson, T. W. and Paulson, T. (2017). *Eurycea aquatica* (Brown-backed Salamander) Nesting. *Herpetological Review*, 48(3):598. 57

[143] Pritchard, J. K., Stephens, M., and Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155(2):945–959. 38

[144] R Core Team (2019). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. 17, 18, 39, 40, 48, 49, 50

[145] Ramsey, J. (1965). *Zoogeographic studies of the freshwater fish fauna of rivers draining the southern Appalachian region*. PhD thesis, Tulane University. 14, 25, 28

[146] Rand, D. M. and Harrison, R. G. (1989). Ecological genetics of a mosaic hybrid zone: mitochondrial, nuclear, and reproductive differentiation of crickets by soil type. *Evolution*, 43(2):432–449. 4

[147] Reagan, N. L. (1984). Courtship behavior of the *Eurycea bislineata wilderae* complex. Master's thesis, Western Carolina University. 9, 45, 50, 55

[148] Rissler, L. J. and Apodaca, J. J. (2007). Adding more ecology into species delimitation: ecological niche models and phylogeography help define cryptic species in the black salamander (*Aneides flavipunctatus*). *Systematic Biology*, 56(6):924–942. 2

[149] Rissler, L. J. and Smith, W. H. (2010). Mapping amphibian contact zones and phylogeographical break hotspots across the United States. *Molecular Ecology*, 19(24):5404–5416. 15

[150] Rose, F. L. and Bush, F. M. (1963). A new species of *Eurycea* (Amphibia: Caudata) from the southeastern United States. *Tulane Studies in Zoology*, 10(2):121–128. 8, 12

[151] Ross, R. (1971). The drainage history of the Tennessee River. In Holt, P. C., Parker, B. C., and Roane, M. K., editors, *The distributional history of the biota of the southern Appalachians*, volume III: Vertebrates, pages 11–42. Virginia Polytechnic Institute and State University. 14, 27, 28

[152] Ross, R. D. (1969). Drainage evolution and fish distribution problems in the southern Appalachians of Virginia. In Holt, P. C., Parker, B. C., and Roane, M. K., editors, *The*

distributional history of the biota of the southern Appalachians, volume I: Invertebrates, pages 277–292. Virginia Polytechnic Institute and State University. 13, 24

[153] Schliep, K., Potts, A. J., Morrison, D. A., and Grimm, G. W. (2017). Intertwining phylogenetic trees and networks. *Methods in Ecology and Evolution*, 8(10):1212–1220. 17

[154] Schliep, K. P. (2011). `phangorn`: phylogenetic analysis in `R`. *Bioinformatics*, 27(4):592–593. 17

[155] Sepkoski Jr, J. J. and Rex, M. A. (1974). Distribution of freshwater mussels: coastal rivers as biogeographic islands. *Systematic Biology*, 23(2):165–188. 13

[156] Sessions, S. K. (1996). Chromosomes: Molecular cytogenetics. In Hillis, D. and Moritz, C., editors, *Molecular Systematics*. Sinaeur, 2nd edition. 49

[157] Sessions, S. K. and Wiktorowski, J. L. (2000). Population cytogenetics of the plethodontid salamander *Eurycea wilderae*. In *The Biology of Plethodontid Salamanders*, pages 327–343. Springer. 47, 56, 57, 106

[158] Sever, D. M. (1972). Geographic variation and taxonomy of *Eurycea bislineata* (Caudata: Plethodontidae) in the upper Ohio River Valley. *Herpetologica*, 28:314–324. 11, 44

[159] Sever, D. M. (1975). Morphology and seasonal variation of the nasolabial glands of *Eurycea quadridigitata* (Holbrook). *Journal of Herpetology*, 9:337–348. 44

[160] Sever, D. M. (1979a). Male secondary sexual characters of the *Eurycea bislineata* (Amphibian, Urodela, Plethodontidae) complex in the sourthern Appalachian Mountains. *Journal of Herpetology*, 13(3):245–253. 8, 9, 27, 45, 46, 54

[161] Sever, D. M. (1979b). Observations on the nasolabial groove of the plethodontid salamander *Eurycea quadridigitata*. *Proceedings of the Indiana Academy of Science*, 89:421–424. 44

[162] Sever, D. M. (1989). Comments on the taxonomy and morphology of two-lined salamanders of the *Eurycea bislineata* complex. *Bulletin of the Chicago Herpetological Society*, 24(4):70–74. 8, 11, 58

[163] Sever, D. M. (1999). *Eurycea bislineata*. *Catalogue of American Amphibians and Reptiles*. 8

[164] Sever, D. M., Dundee, H. A., and Sullivan, C. D. (1976). A new *Eurycea* (Amphibia: Plethodontidae) from southwestern North Carolina. *Herpetologica*, pages 26–29. 8, 26, 32

[165] Sever, D. M., Siegel, D. S., Taylor, M. S., and Beachy, C. K. (2016). Phylogeny of mental glands, revisited. *Copeia*, 104(1):83–93. 44

[166] Shen, X.-X., Liang, D., Chen, M.-Y., Mao, R.-L., Wake, D. B., and Zhang, P. (2015). Enlarged multilocus data set provides surprisingly younger time of origin for the Plethodontidae, the largest family of salamanders. *Systematic Biology*, 65(1):66–81. 1

[167] Shimodaira, H. and Hasegawa, M. (1999). Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution*, 16(8):1114–1114. 17

[168] Sidiropoulos, N., Sohi, S. H., Pedersen, T. L., Porse, B. T., Winther, O., Rapin, N., and Bagger, F. O. (2018). SinaPlot: an enhanced chart for simple and truthful representation of single observations over multiple classes. *Journal of Computational and Graphical Statistics*, 27(3):673–676. 50

[169] Smith, T. B. and Skúlason, S. (1996). Evolutionary significance of resource polymorphisms in fishes, amphibians, and birds. *Annual Review of Ecology and Systematics*, 27(1):111–133. 7

[170] Sota, T. and Kubota, K. (1998). Genital lock-and-key as a selective agent against hybridization. *Evolution*, 52(5):1507–1513. 5

[171] Stamatakis, A. (2014). `RAxML` version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30(9):1312–1313. 17, 47

[172] Starnes, W. and Etnier, D. (1986). Drainage evolution and fish biogeography of the Tennessee and Cumberland Rivers drainage realm. In Hocutt, C. and Wiley, E., editors, *The Zoogeography of North American Freshwater Fishes*, pages 325–361. Wiley. 25, 27

[173] Staub, N. L. (1993). Intraspecific agonistic behavior of the salamander *Aneides flavipunctatus* (Amphibia: Plethodontidae) with comparisons to other plethodontid species. *Herpetologica*, 49:271–282. 58

[174] Stewart, K., Hudson, C., and Lougheed, S. (2017). Can alternative mating tactics facilitate introgression across a hybrid zone by circumventing female choice? *Journal of Evolutionary Biology*, 30(2):412–421. 7

[175] Stoneburner, D. (1978). Salamander drift: Observations on the two-lined salamander (*Eurycea bislineata*). *Freshwater Biology*, 8(3):291–293. 10

[176] Strickland, J. C., Pinheiro, A. P., Cecala, K. K., and Dorcas, M. E. (2016). Relationship between behavioral thermoregulation and physiological function in larval stream salamanders. *Journal of Herpetology*, 50(2):239–244. 34

[177] Swenson, N. G. and Howard, D. J. (2005). Clustering of contact zones, hybrid zones, and phylogeographic breaks in North America. *The American Naturalist*, 166(5):581–591. 1

[178] Taborsky, M. (1997). Bourgeois and parasitic tactics: do we need collective, functional terms for alternative reproductive behaviours? *Behavioral Ecology and Sociobiology*, 41(5):361–362. 6, 7

[179] Taylor, S. A. and Larson, E. L. (2019). Insights from genomes into the evolutionary importance and prevalence of hybridization in nature. *Nature Ecology & Evolution*, 3(2):170. 1, 2, 29, 31

[180] Thoma, R. F. (2005). *Cambarus* (*Cambarus*) *eeseeohensis* (Decapoda: Cambaridae), a new species of crayfish from the Linville River of North Carolina and its bearing on understanding the evolution of the subgenus *Cambarus*. *Proceedings of the Biological Society of Washington*, 118(4):794–803. 28

[181] Thomas, J. S. (1989). *Courtship, male-male competition, and male aggressive behavior of the salamander* Eurycea bislineata. PhD thesis, University of Southwestern Louisiana. 45

[182] Timpe, E. K., Graham, S. P., and Bonett, R. M. (2009). Phylogeography of the brownback salamander reveals patterns of local endemism in southern Appalachian springs. *Molecular Phylogenetics and Evolution*, 52(2):368–376. 8, 26, 32

[183] Tryon, G. W. (1873). *Land and fresh-water shells of North America*. Smithsonian Institution. 14

[184] Tuttle, E. M., Bergland, A. O., Korody, M. L., Brewer, M. S., Newhouse, D. J., Minx, P., Stager, M., Betuel, A., Cheviron, Z. A., Warren, W. C., Gonser, R. A., and Balakrishnan, C. N. (2016). Divergence and functional degradation of a sex chromosome-like supergene. *Current Biology*, 26(3):344–350. 47

[185] Twyford, A. and Ennos, R. (2012). Next-generation hybridization and introgression. *Heredity*, 108(3):179. 1

[186] Usuda, H. (1997). Individual relationship of male aggressive behavior during the reproductive season of *Hynobius nigrescens*. *Japanese Journal of Herpetology*, 17:53–61. 7

[187] Verrell, P. A. (1990a). Frequency of interspecific mating in salamanders of the plethodontid genus *Desmognathus*: different experimental designs may yield different results. *Journal of Zoology*, 221(3):441–451. 6, 44

[188] Verrell, P. A. (1990b). Sexual compatibility among plethodontid salamanders: tests between *Desmognathus apalachicolae*, and *D. ochrophaeus* and *D. fuscus*. *Herpetologica*, pages 415–422. 6, 44

[189] Verrell, P. A. and Arnold, S. J. (1989). Behavioral observations of sexual isolation among allopatric populations of the mountain dusky salamander, *Desmognathus ochrophaeus*. *Evolution*, 43(4):745–755. 6

[190] Verrell, P. A. and Tilley, S. G. (1992). Population differentiation in plethodontid salamanders: divergence of allozymes and sexual compatibility among populations of *Desmognathus imitator* and *D. ochrophaeus* (Caudata: Plethodontidae). *Zoological Journal of the Linnean Society*, 104(1):67–80. 6, 44

[191] Wake, D. B. (2006). Problems with species: patterns and processes of species formation in salamanders. *Annals of the Missouri Botanical Garden*, 93(1):8–23. 2

[192] Wake, D. B. (2009). What salamanders have taught us about evolution. *Annual Review of Ecology, Evolution, and Systematics*, 40:333–352. 1

[193] Wake, D. B., Yanev, K. P., and Brown, C. W. (1986). Intraspecific sympatry in a "ring species," the plethodontid salamander *Ensatina eschscholtzii*, in southern California. *Evolution*, 40(4):866–868. 4

[194] Walls, S. C. (2009). The role of climate in the dynamics of a hybrid zone in Appalachian salamanders. *Global Change Biology*, 15(8):1903–1910. 4

[195] Weaver, W. (1897). River adjustments in North Carolina. *Elisha Mitchell Scientific Society Journal*, 13(1):13–24. 27

[196] Weisrock, D., Kozak, K., and Larson, A. (2005). Phylogeographic analysis of mitochondrial gene flow and introgression in the salamander, *Plethodon shermani*. *Molecular Ecology*, 14(5):1457–1472. 3

[197] Weisrock, D. W., Hime, P. M., Nunziata, S. O., Jones, K. S., Murphy, M. O., Hotaling, S., and Kratovil, J. D. (2018). Surmounting the large-genome "problem" for genomic data generation in salamanders. In *Population Genomics*. Springer. 35

[198] Wells, K. D. (1977). The social behaviour of anuran amphibians. *Animal Behaviour*, 25:666–693. 6, 7

[199] White, C. H. (1904). The Appalachian River versus a Tertiary trans-Appalachian river in eastern Tennessee. *The Journal of Geology*, 12(1):34–39. 14

[200] White, W. A. (1953). Systematic drainage changes in the Piedmont of North Carolina and Virginia. *Bulletin of the Geological Society of America*, 64(561-580). 12

[201] Wiens, J. J. and Graham, C. H. (2005). Niche conservatism: integrating evolution, ecology, and conservation biology. *Annual Review of Ecology, Evolution, and Systematics*, 36:519–539. 4

[202] Wilbur, H. M. and Collins, J. P. (1973). Ecological aspects of amphibian metamorphosis: nonnormal distributions of competitive ability reflect selection for facultative metamorphosis. *Science*, 182(4119):1305–1314. 7

[203] Willett, S. D., McCoy, S. W., Perron, J. T., Goren, L., and Chen, C.-Y. (2014). Dynamic reorganization of river basins. *Science*, 343(6175):1248765. 29

[204] Wirtz, P. (1999). Mother species–father species: unidirectional hybridization in animals with female choice. *Animal Behaviour*, 58(1):1–12. 4

[205] Wood, R. M. and Mayden, R. L. (1992). Systematics, evolution, and biogeography of *Notropis chlorocephalus* and *N. lultipinnis*. *Copeia*, 1992(1):68–81. 13

[206] Wooten, J. A., Camp, C. D., Combs, J. R., Dulka, E., Reist, A., and Walker, D. M. (2013). Re-evaluating niche conservatism versus divergence in the woodland salamander genus *Plethodon*: a case study of the parapatric members of the *Plethodon glutinosus* species complex. *Canadian Journal of Zoology*, 91(12):883–892. 5

[207] Wray, K. P. and Steppan, S. J. (2017). Ecological opportunity, historical biogeography and diversification in a major lineage of salamanders. *Journal of Biogeography*, 44(4):797–809. 11, 19, 23, 34

[208] Yerger, R. and Relyea, K. (1968). The flat-headed bullheads (Pisces: Ictaluridae) of the southeastern United States, and a new species of *Ictalurus* from the Gulf Coast. *Copeia*, 1968(2):361–384. 13

[209] Zamudio, K. and Chan, L. (2008). Alternative reproductive tactics in amphibians. In Oliveira, R. F., Taborsky, M., and Brockmann, H. J., editors, *Alternative reproductive tactics, an integrative approach*, pages 300–331. Cambridge University Press. 7

Appendix

A Tables

Table 1: Results of Shimodaira–Hasegawa tests. The first column refers to the 3RAD assembly used for each test. The second column refers to the 3RAD assembly from which I originally inferred the maximum-likelihood topology, and the second column refers to the 3RAD assembly from which I originally inferred the alternative topology. In the first two examples, I created this alternative topology by pruning the *full_min5* tree to retain only taxa in the northern or southern clade, respectively. In the third example, I created the alternative topology by combining the topologies of the trees inferred from the *north* and *south* assemblies. P-values are in reference to the null hypothesis, which is that the both topologies have the same likelihood.

Assembly	**ML Topol.**	**Alt. Topol.**	**Best Topol. Lik.**	**Alt. Topol. Lik.**	**Δ Lik.**	**P-value**
north	*north*	*full_min5* (northern)	-619690.22	-620102.21	-411.99	< 0.01
south	*south*	*full_min5* (southern)	-748612.11	-748941.42	-329.30	< 0.01
full_min5	*full_min5*	*north* + *south*	-1187522.63	-1189966.56	-2443.93	< 0.01

Table 2: D-statistics for select taxa in the *Eurycea bislineata* species complex. Labels used in the topology reflect lineage names from Kozak et al. [106] and are explained in greater detail in the Methods. The topologies reflect the maximum-likelihood phylogeny inferred from the *north* or *south* assemblies. P-values are in reference to the null hypothesis, which is that D = 0, and were calculated using Z-scores from 100 bootstrap replicates.

Topology	D	Z-score	P-value
(((S, E1), KL*), out)	-0.23	5.61	< 0.01
(((E2, E1), KL*), out)	-0.14	4.66	< 0.01
(((I, E1), KL*), out)	-0.10	2.54	0.01
(((J, M), AB), out)	-0.13	6.25	< 0.01
(((GH, CF), KL*), out)	-0.06	2.76	< 0.01

Table 3: Migration model comparison using coalescent simulations in `fastsimcoal2`. Labels used in the topology reflect lineage names from Kozak et al. [106] and are explained in greater detail in the Methods. The topology column reflects the order of coalescent events in the models, and the migration column reflects the direction(s) of migration allowed in each model. Note that the direction of this migration is in a coalescent framework (e.g., migration from E2→J reflects migration from J→E2 in forward-time). Columns 3–9 show parameter estimates from models, and Columns 10–12 show likelihoods, AIC values (for the best model), and ΔAIC scores (for all other models). The best model for each set of taxa is shown in bold.

			Gener. (x 10^6)				**Mig. Weight**				
Topology	Mig.	Pop.	Mig.	1	2	3	1→4	1←4	Lik.	AIC	ΔAIC
(((AB, E1), E2), J)	-	3237259	-	2.7	2.9	3.4	-	-	-1338.2	-	153
(((AB, E1), E2), J)	E2→J	2472994	0.8	2.5	2.6	3.4	0.24	-	-1321.106	-	78
(((AB, E1), E2), J)	E2↔J	2040622	1.0	2.1	2.1	3.4	0.30	0.13	-1320.2	-	76
(((E2, E1), AB), J)	-	2987262	-	2.1	3.3	3.4	-	-	-1329.4	-	112
(((E2, E1), AB), J)	E2→J	2318737	1.2	1.3	2.8	3.4	0.33	-	-1307.5	-	16
(((E2, E1), AB), J)	**E2↔J**	**1933496**	**1.1**	**1.1**	**2.5**	**3.4**	**0.36**	**0.17**	**-1303.7**	**6017**	**-**
(((AB, CF), GH), KL*)	-	1364140	-	1.3	1.3	3.4	-	-	-1505.6	-	60
(((AB, CF), GH), KL*)	GH→KL*	1333514	0.5	1.3	1.3	3.4	0.03	-	-1503.3	-	53
(((AB, CF), GH), KL*)	GH↔KL*	1331175	0.7	1.3	1.3	3.4	0.03	0.00	-1503.5	-	56
(((GH, CF), AB), KL*)	-	1335946	-	0.9	1.5	3.4	-	-	-1495.2	-	12
(((GH, CF), AB), KL*)	**GH→KL***	**1244498**	**0.5**	**0.9**	**1.5**	**3.4**	**0.04**	**-**	**-1491.8**	**6881**	**-**
(((GH, CF), AB), KL*)	GH↔KL*	1237555	0.7	0.9	1.5	3.4	0.06	0.00	-1491.8	-	2

Table 4: Karyotypes for select *Eurycea* cf. *wilderae*. ST = subtelocentric; T = telocentric.

Locality	**Lineage**	**Individual**	**Morph**	**Karyotype**
Paint Creek	Lineage J	LHUP 1763	searching	T/T
		LHUP 1762	Morph A	T/T
		LHUP 1761	Morph A	T/T
		LHUP 1764	searching	T/T
Davenport Gap	Lineage M	LHUP 1766	searching	T/T
		LHUP 1765	Morph A	T/T
Cripple Creek	Lineage M	LHUP 1769	searching	T/T
		LHUP 1767	Morph A	T/T

Table 5: Results of preliminary field surveys for *Eurycea* cf. *wilderae*, conducted in Great Smoky Mountains National Park from 2017–2018.

		15-Oct	**23-Oct**	**28-Oct**	**6-Nov**	**15-Nov**	**30-Nov**	**5-Dec**	**11-Jan**	**Total**
forest	Morph A ♂	0	0	0	1	0	0	1	0	2
	searching ♂	2	11	0	6	1	1	10	2	33
	gravid ♀	4	7	3	17	0	2	6	0	39
	juv./non-rep.	1	0	0	6	0	2	30	0	39
stream	Morph A ♂		0	0	1	1	1	0	2	5
	searching ♂		0	0	0	0	0	0	5	5
	gravid ♀		0	0	0	1	2	0	4	7
	juv./non-rep.		0	0	0	0	0	0	12	12

Table 6: Observations of *Eurycea* cf. *wilderae* from funnel trap surveys of the forest floor, conducted at Highlands Biological Station in Fall 2018.

	17 Oct	**18 Oct**	**25 Oct**	**26 Oct**	**27 Oct**	**28 Oct**	**02 Nov**	**05 Nov**	**Total**
Morph A ♂	0	0	1	0	0	0	2	0	3
searching ♂	4	0	0	2	1	0	1	0	8
gravid ♀	0	1	0	0	1	1	1	1	5
juv./non-rep.	0	0	0	1	0	0	1	0	2

Table 7: Observations of *Eurycea* cf. *wilderae* from nocturnal forest floor surveys, conducted at Highlands Biological Station in Fall 2018.

	16 Oct	**17 Oct**	**18 Oct**	**26 Oct**	**01 Nov**	**05 Nov**	**09 Nov**	**Total**
Morph A ♂	0	0	0	0	0	0	0	0
searching ♂	6	0	0	12	17	22	14	71
gravid ♀	4	1	0	8	8	3	1	25
juv./non-rep.	21	0	2	14	19	25	9	90

Table 8: Observations of *Eurycea* cf. *wilderae* from diurnal stream surveys, conducted at Highlands Biological Station in Fall 2018.

	17 Oct	**18 Oct**	**19 Oct**	**20 Oct**	**23 Oct**	**24 Oct**	**11 Nov**	**Total**
Morph A ♂	9	5	11	3	0	1	17	46
searching ♂	0	0	0	0	0	0	3	3
gravid ♀	3	3	9	2	6	0	24	47
juv./non-rep.	0	0	2	0	1	1	22	26

B Figures

Figure 1: Recognized taxa in the *Eurycea bislineata* species complex.

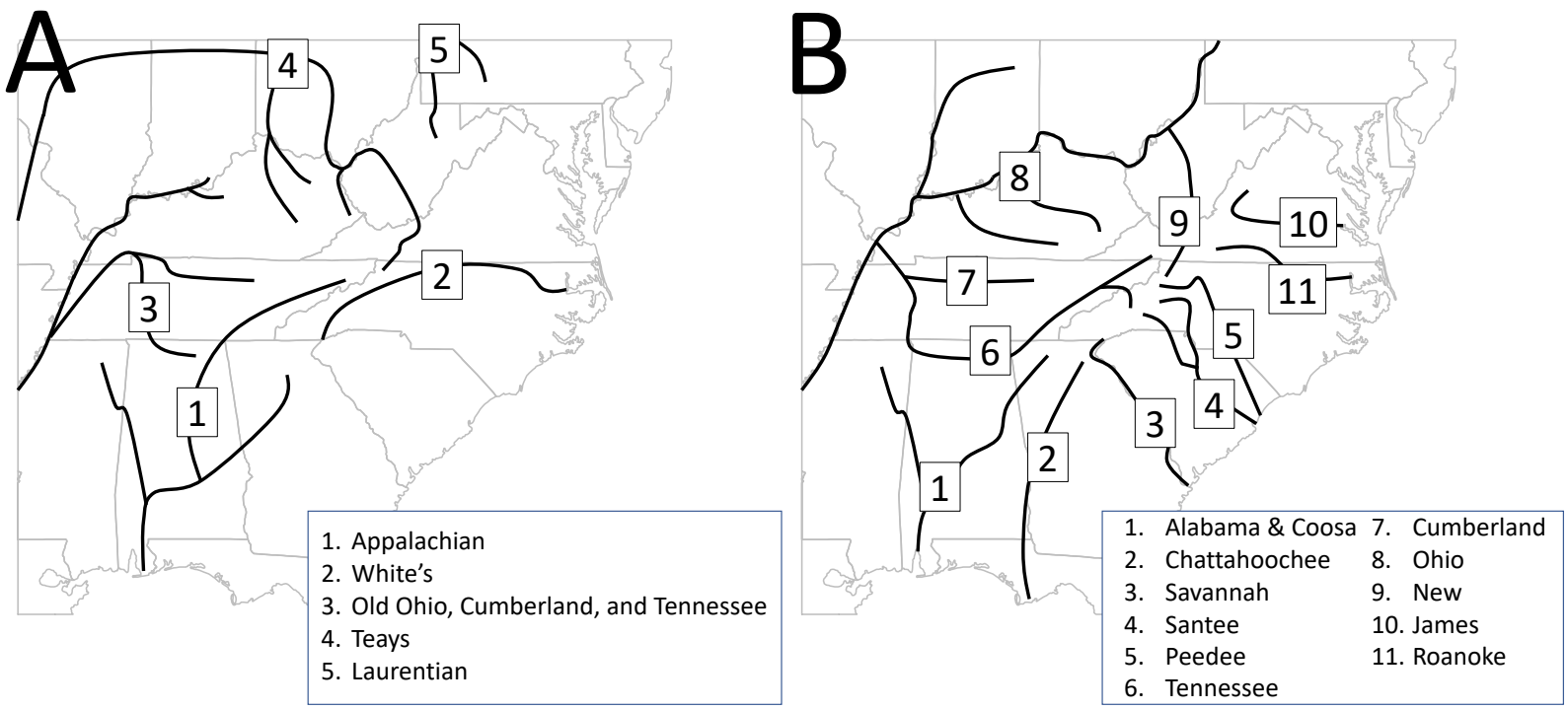

Figure 2: Maps of the eastern United States showing A) hypothesized paleodrainages; and B) major modern river drainages. See the Introduction for more detail.

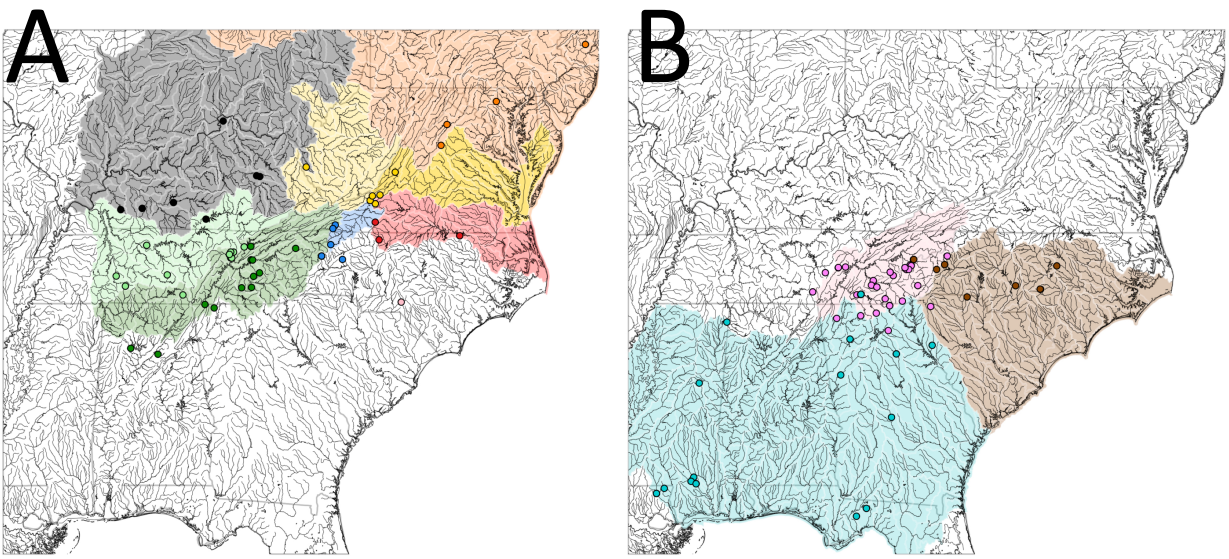

Figure 3: Maps of sampling localities, with colors corresponding to major mitochondrial and genomic lineages and corresponding river drainages. A) "northern" clade; B) "southern" clade. Black = Muskingum, Scioto, Great Miami, Middle Ohio, Kentucky-Licking, Green, Wabash, and Ohio River drainages; Light green = Cumberland, Middle Tennessee-Elk, and Tennessee drainages; Dark green = Upper Tennessee, Middle Tennessee-Hiwassee, and Middle Tennessee-Elk River drainages; Orange = Upper Hudson, Hudson-Long Island, Delaware-Mid Atlantic Coastal, Susquehanna, Upper Chesapeake, Potomac, Western Lake Erie, Southern Lake Erie, Allegheny, Monongahela, and portions of the Upper Ohio River drainages; Light yellow = Big Sandy-Guyandotte, portions of the Upper Ohio, and portions of the Kanawha River drainage; Dark yellow = Chesapeake and James River drainages; Royal blue = Upper New River drainage; Red = Chowan-Roanoke River drainage; Pink = Upper Tennessee and Middle Tennessee-Hiwassee River drainages; Brown = Neuse-Pamlico, Cape Fear, Pee Dee, and Edisto-Santee River drainages; Turquoise = Ogeechee-Savannah, Altamaha-St. Marys, Suwannee, Ochlockonee, Apalachicola, Choctawhatchee-Escambia, Alabama, Mobile-Tombigbee, Pascagoula, Pearl, Mississippi-Yazoo, and Mississippi-Big Black River drainages. See Figure S1 for a version of this map with sample labels included.

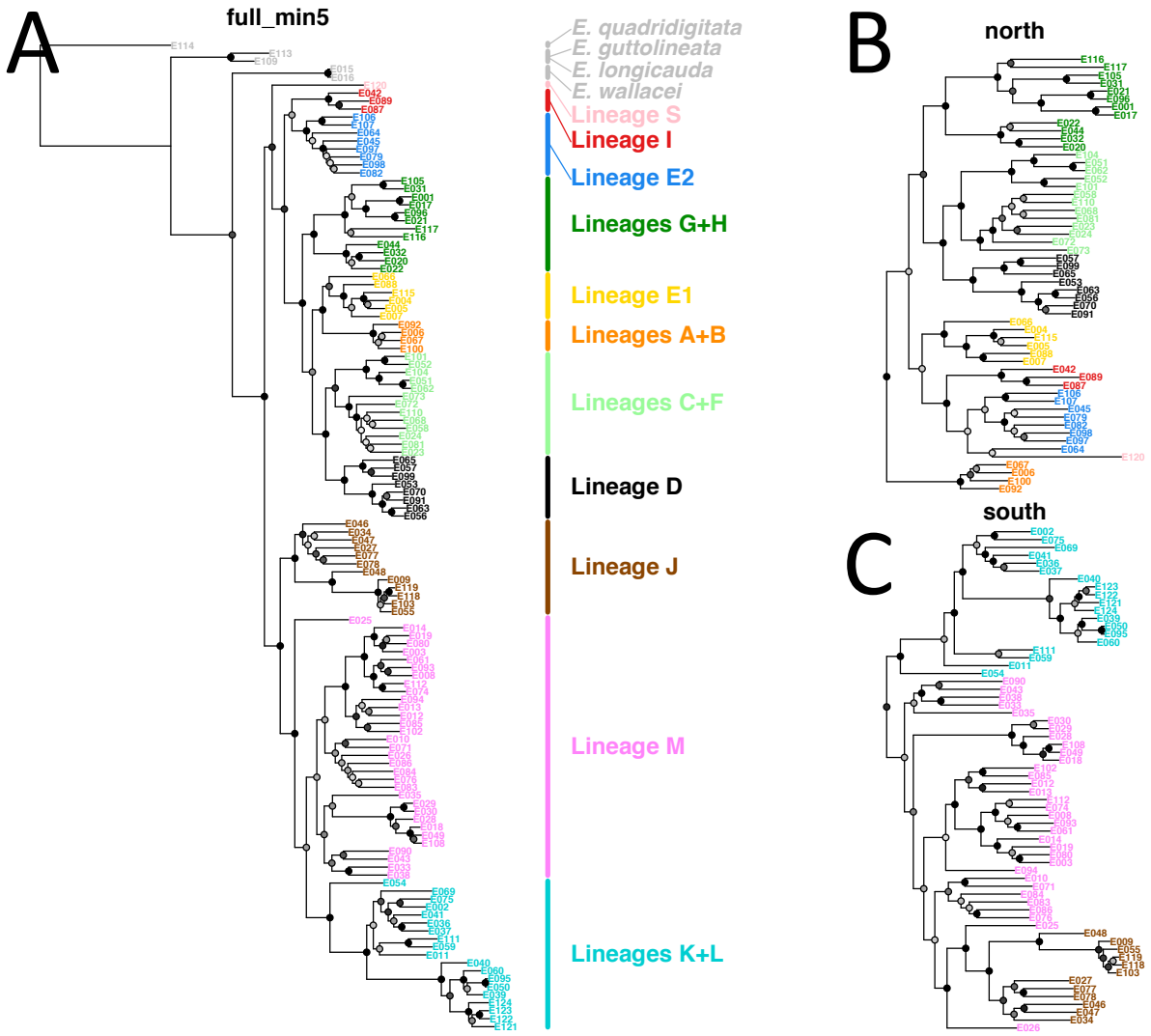

Figure 4: Maximum-likelihood phylogenies estimated from three 3RAD SNP datasets. A) *full_min5*, which includes all samples and all SNPs in loci shared in at least five samples; B) *north*, which includes only samples from the northern clade plus outgroups; and C) *south*, which includes only samples from the southern clade plus outgroups. Although I inferred these latter two phylogenies rooted with outgroups, I have pruned the phylogenies to omit them for this figure. Lineage labels and colors correspond to major mtDNA lineage names from Kozak et al. [106] and explained in the Methods, and continuous variation in node color represents bootstrap edge support from 0 (white) to 100 (black).

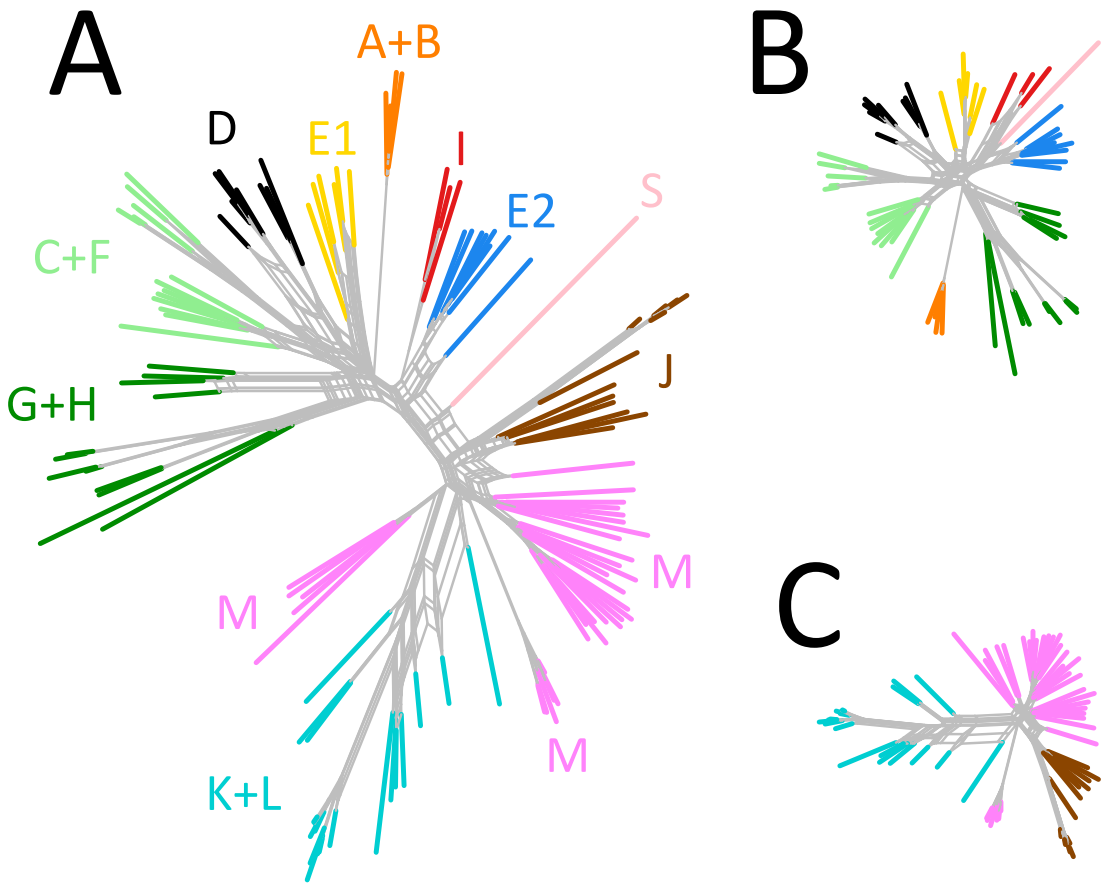

Figure 5: Distance-based phylogenetic networks estimated from the *full_min30* assembly. A) all samples; B) only samples from the northern clade; and C) only samples from the southern clade. Colors correspond to major lineages as explained in the Results. See Figure S3 for a version of this figure with tip labels included.

.

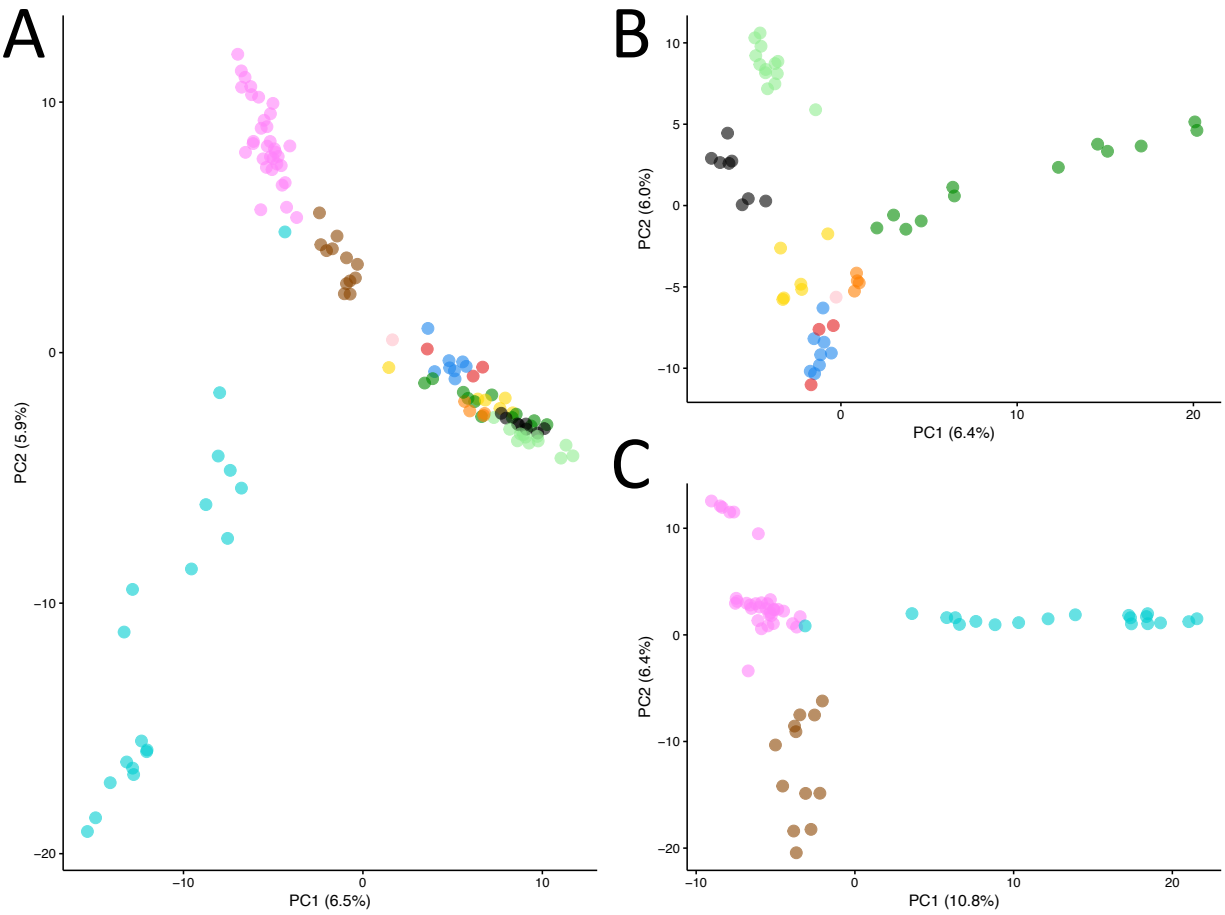

Figure 6: Principal components analyses from the *full_min5* assembly. A) all samples and all SNPs; B) only samples from the northern clade; and C) only samples from the southern clade. Colors correspond to major lineages as explained in the Results. See Figure S4 for a version of this figure with sample labels included.

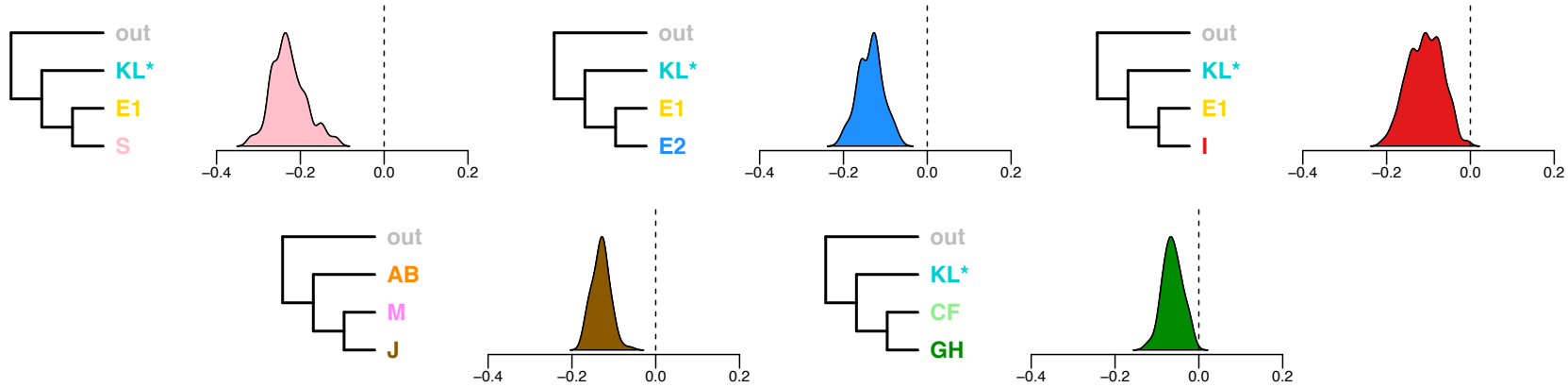

Figure 7: D-statistic tests for introgression among major lineages in the *Eurycea bislineata* species complex. Distributions show bootstrap replicates. Colors correspond to major lineages as explained in the Results.

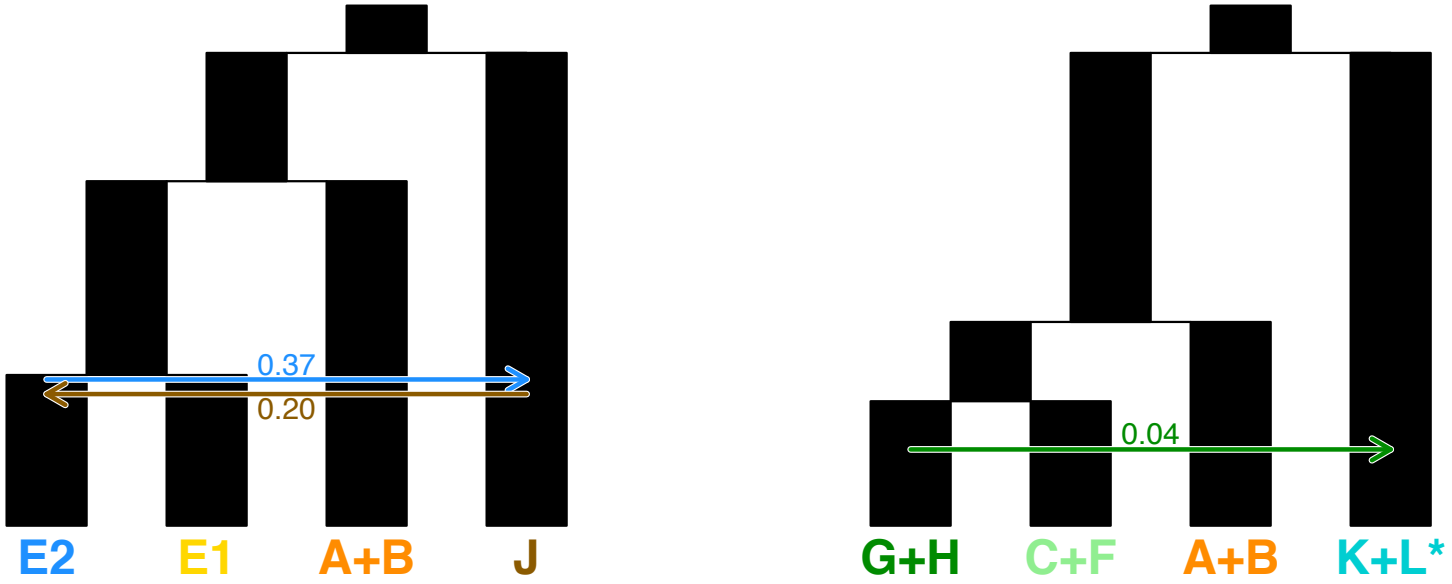

Figure 8: Best migration models as inferred from coalescent simulations in `fastsimcoal2`. Arrows represents migration in a coalescent framework (i.e., the reverse of forward-time), and are labeled with migration estimates. Bar heights represent relative coalescent times. Colors correspond to major lineages as explained in the Results.

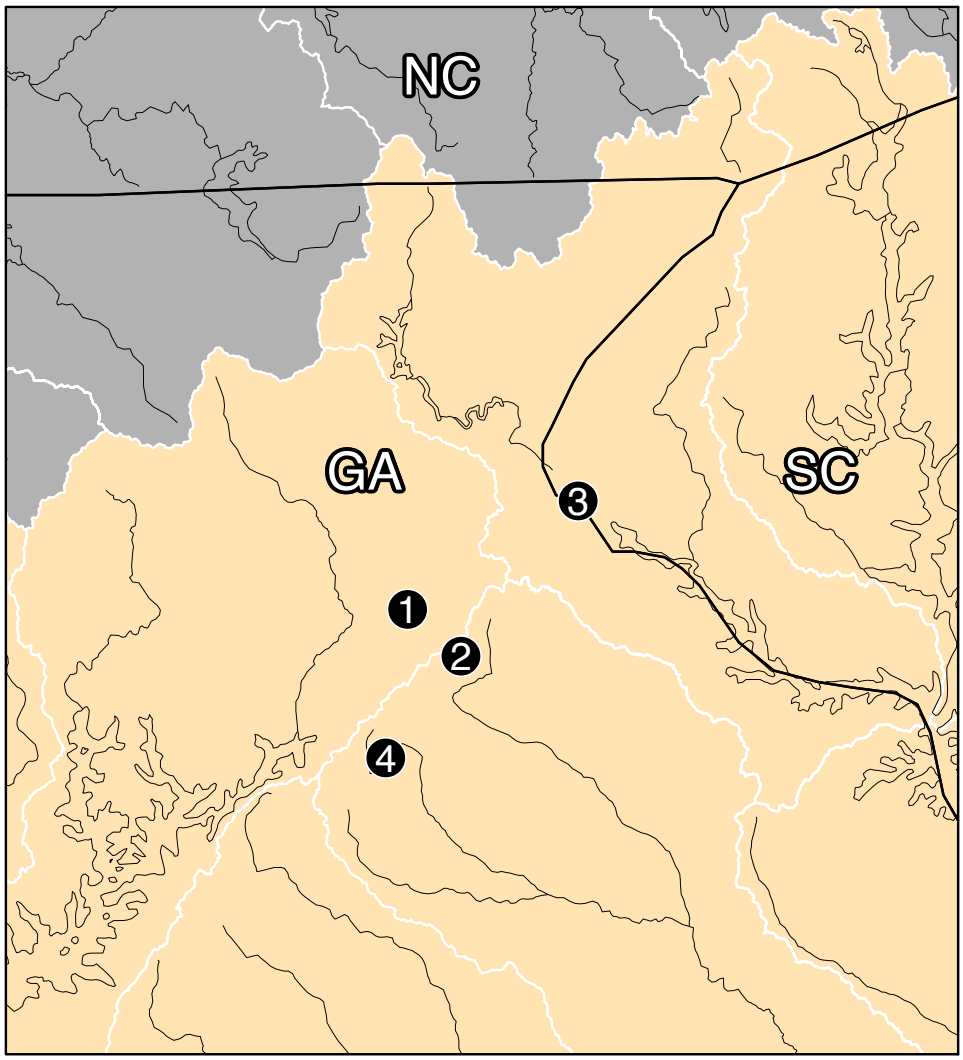

Figure 9: Map of replicate contact zones. 1 = Unnamed tributary of Camp Creek (Chattahoochee); 2 = Nancy Town Creek (Savannah); 3 = Rothwell Creek (Savannah); 4 = Unnamed tributary of Hudson River (Savannah). Gray shading represents the Tennessee River Drainage, and orange shading represents all other river drainages.

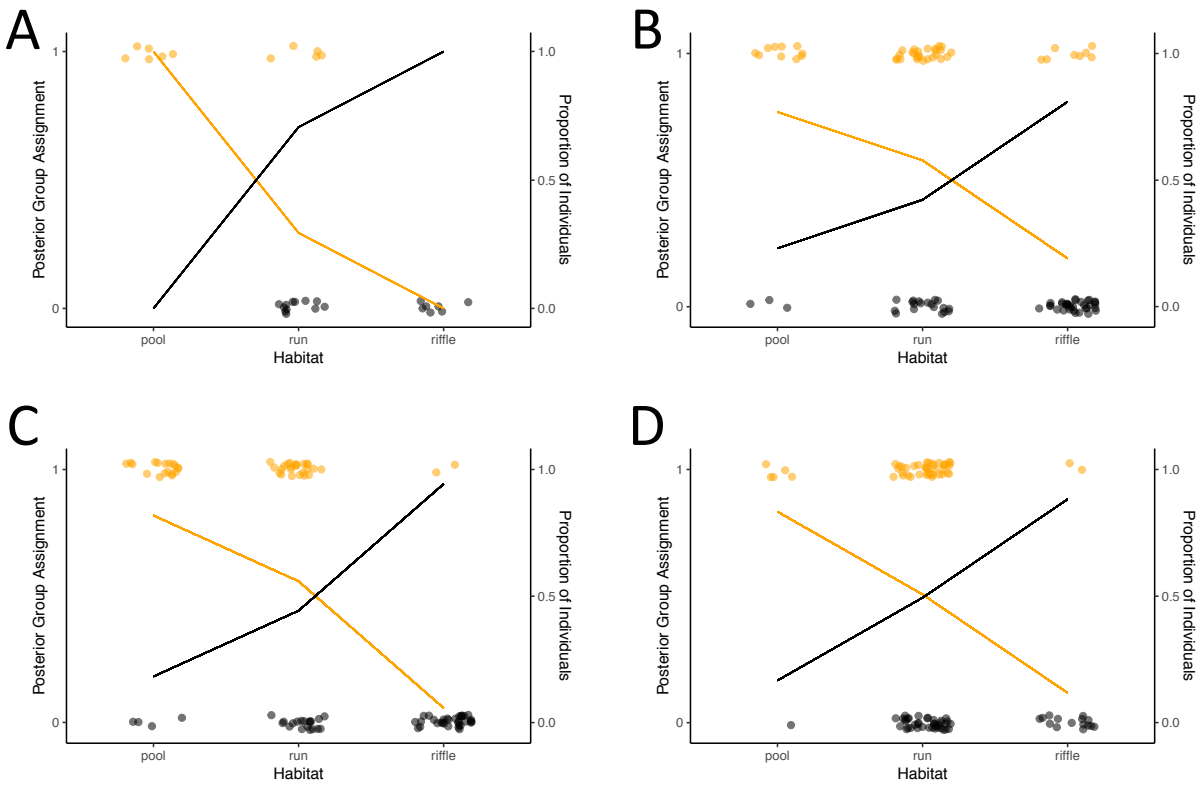

Figure 10: Stream habitat vs. species assignment for Streams 1–4 (A–D). Posterior membership probabilities are derived from DAPC results and are vertically jittered for ease of visualization. Lines show the relative frequencies of each species in each habitat. Orange dots and lines represent *E. cirrigera*, and black dots and lines represent *E.* cf. *wilderae*.

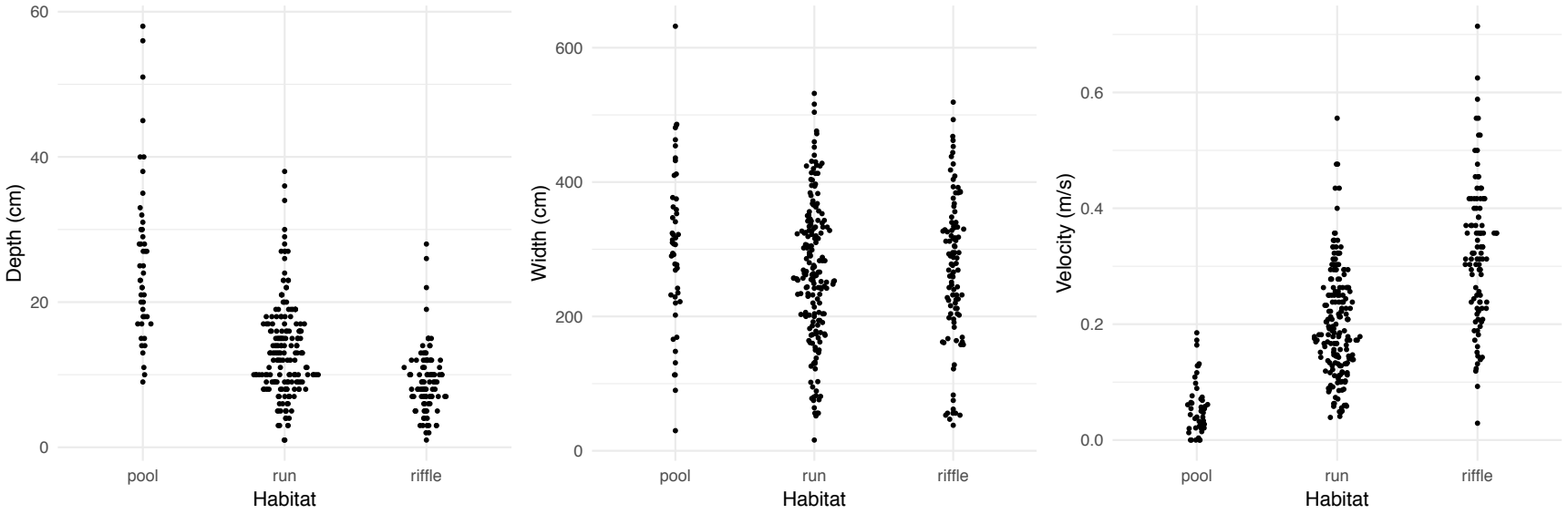

Figure 11: SinaPlots of maximum stream depth, stream width, and stream velocity of three habitats, pooled across all four sites.

Figure 12: Dorsal (top) and ventral (bottom) views of searching (left) and Morph A (right) male *Eurycea* cf. *wilderae* showing secondary sexual characters. The short arrows point to cirri, and the long arrow points to the mental gland on a searching male.

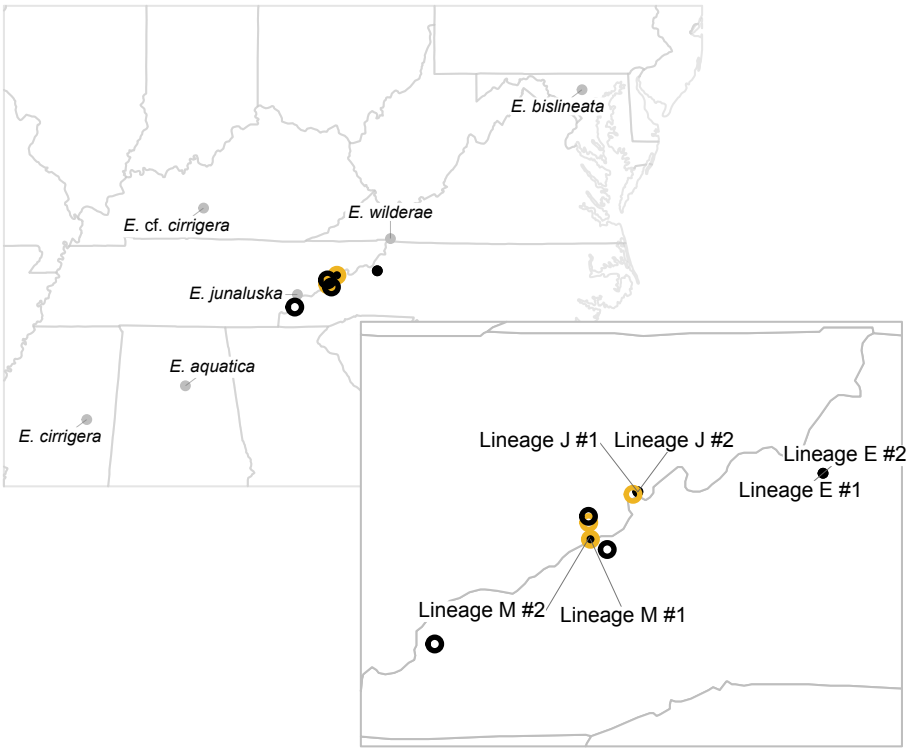

Figure 13: Sampling map for alternative reproductive tactics. Black and gray dots represent localities for paired samples and reference samples, respectively, in the phylogeny. Open circles represent localities from which *Eurycea* were collected for behavioral trials, with orange circles indicating the subset of these populations from which individuals were used in the karyological study. For labeled samples from the phylogeny, those labeled "#1" represent searching males, and those labeled "#2" represent Morph A males

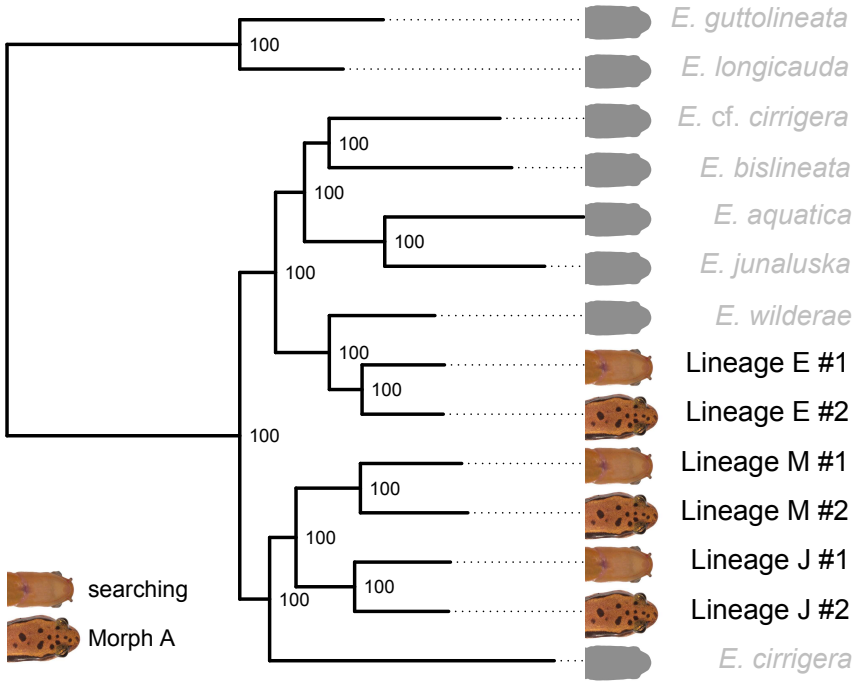

Figure 14: Maximum-likelihood phylogeny of 3RAD data. Edge labels indicate bootstrap support. In all cases, samples labeled "#1" represent searching males, and those labeled "#2" represent Morph A males.

Figure 15: Binary response of presence of "bite" behavior in mate-guarding trials, pooled across sampling localities.

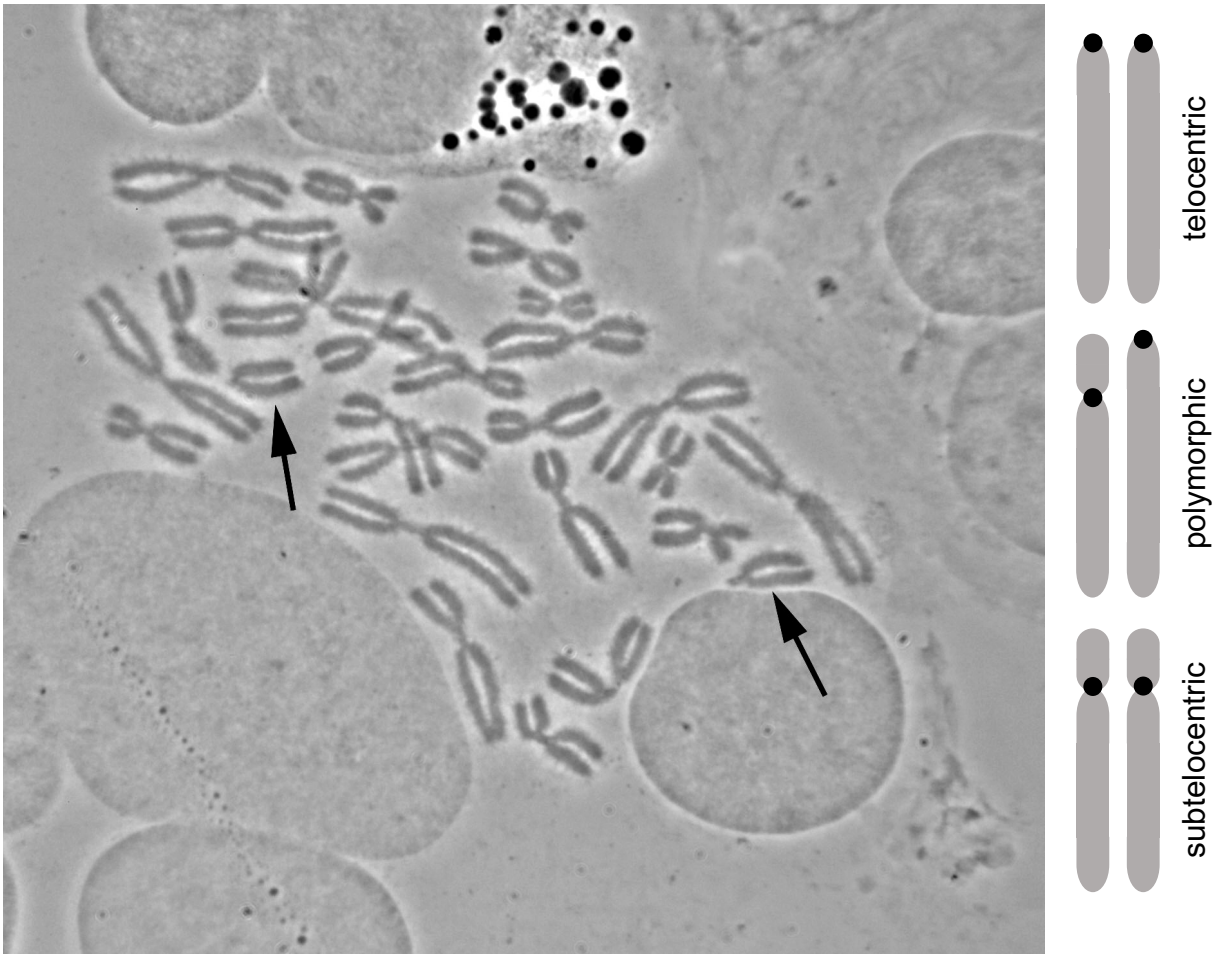

Figure 16: Representative chromosome spread from one *E.* cf. *wilderae*. Arrows indicate the thirteenth chromosomes with telocentric inversions. Reference diagram of three possible karyomorphs (telocentric, subtelocentric, and polymophic) redrawn with permission from Sessions and Wiktorowski [157]. For images of chromosome spreads of all three karyomorphs, see this same publication.

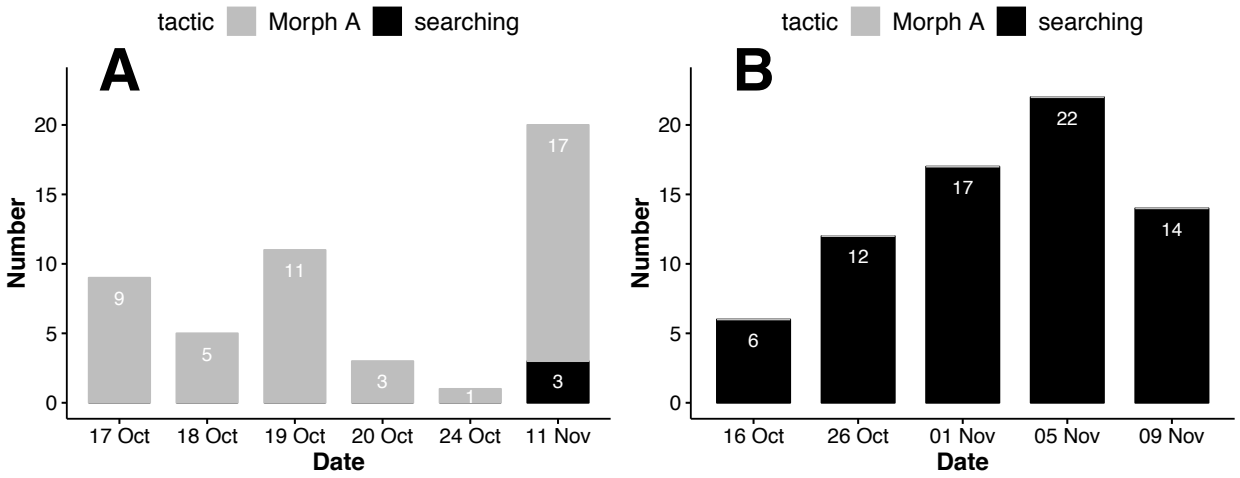

Figure 17: Observations of adult male *Eurycea* cf. *wilderae* from A) stream surveys; and B) forest floor surveys. Data are only shown for nights in which I observed at least one male.

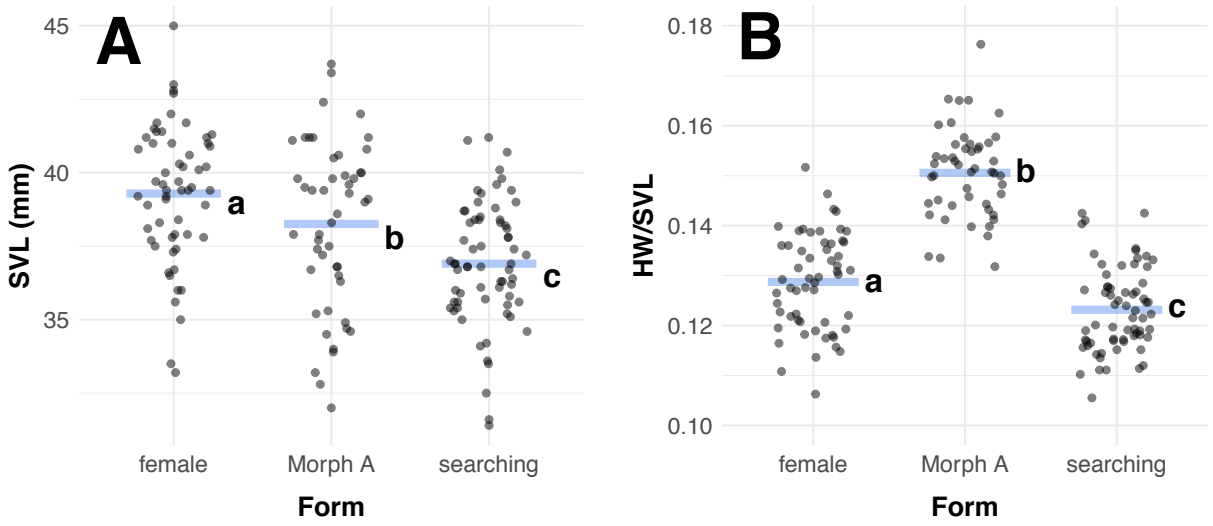

Figure 18: SinaPlots of A) snout-vent length (SVL); and B) head width (HW):SVL ratios of female, Morph A male, and searching male forms of *Eurycea* cf. *wilderae*. Dots represent individual salamanders, and thick blue lines represent means.

C Supplemental Tables

Table S1: Sampling localities for phylogeographic analyses using 3RAD data. Adapter and primer names correspond to unique indexes as detailed in Bayona-Vásquez et al. [11].

Sample Name	Catalog Number	Latitude	Longitude	Left Adapter	Right Adapter	iTru7	iTru5
E001	TWP_224	35.59234	-83.85258	NheI_B	EcoRI_02	iTru7_06_02	iTru7_02_B
E002	TWP_096	33.890974	-83.362278	NheI_C	EcoRI_03	iTru7_06_03	iTru7_02_C
E003	TWP_244	35.357547	-83.917827	NheI_E	EcoRI_04	iTru7_06_04	iTru7_02_D
E004	TWP_262	37.185718	-80.376586	NheI_F	EcoRI_06	iTru7_06_06	iTru7_02_F
E005	TWP_264	37.257174	-80.524793	NheI_G	EcoRI_07	iTru7_06_07	iTru7_02_G
E006	TWP_265	39.42884	-76.923228	NheI_H	EcoRI_08	iTru7_06_08	iTru7_02_H
E007	TWP_266	37.356636	-80.460965	NheI_A	EcoRI_09	iTru7_06_09	iTru5_01_A
E008	TWP_272	34.40271	-83.592145	NheI_B	EcoRI_10	iTru7_06_10	iTru5_01_B
E009	336	35.3872	-79.9622	NheI_C	EcoRI_11	iTru7_06_11	iTru5_01_C
E010	TWP_036	35.61	-83.45	NheI_D	EcoRI_12	iTru7_06_12	iTru5_01_D
E011	7.1411	32.5	-83.5	NheI_E	EcoRI_01	iTru7_07_01	iTru5_01_E
E012	7.109	34.9314	-82.3832	NheI_F	EcoRI_02	iTru7_07_02	iTru5_01_F
E013	8.172	35.39	-82.75	NheI_G	EcoRI_03	iTru7_07_03	iTru5_01_G
E014	8.288	35.5	-84.02	NheI_H	EcoRI_04	iTru7_07_04	iTru5_01_H
E015	KW_0963			NheI_B	EcoRI_02	iTru7_07_06	iTru7_07_B
E016	KW_0960			NheI_C	EcoRI_03	iTru7_07_07	iTru7_07_C
E017	TWP_103	35.66914	-83.68214	NheI_E	EcoRI_05	iTru7_07_09	iTru5_07_E
E018	TWP_118	35.7972	-84.8117	NheI_F	EcoRI_06	iTru7_07_10	iTru5_07_F
E019	TWP_121	35.39772	-84.07364	NheI_G	EcoRI_07	iTru7_07_11	iTru5_07_G

Table S1. Continued.

Sample Name	Catalog Number	Latitude	Longitude	Left Adapter	Right Adapter	iTru7	iTru5
E020	TWP_226	36.20596	-82.6508	NheI_H	EcoRI_08	iTru7_07_12	iTru5_07_H
E021	TWP_153	35.34128	-84.19174	NheI_A	EcoRI_09	iTru7_08_01	iTru5_06_A
E022	TWP_155	35.956403	-83.92237	NheI_B	EcoRI_10	iTru7_08_02	iTru5_06_B
E023	TWP_156	36.12485	-84.42295	NheI_C	EcoRI_11	iTru7_08_03	iTru5_06_C
E024	TWP_159	36.12604	-84.50445	NheI_D	EcoRI_12	iTru7_08_04	iTru5_06_D
E025	TWP_167	35.84103	-82.96892	NheI_E	EcoRI_01	iTru7_08_05	iTru5_06_E
E026	TWP_170	35.8253	-82.93752	NheI_F	EcoRI_02	iTru7_08_06	iTru5_06_F
E027	TWP_175	35.96126	-82.87522	NheI_G	EcoRI_03	iTru7_08_07	iTru5_06_G
E028	TWP_179	35.67282	-85.38508	NheI_H	EcoRI_04	iTru7_08_08	iTru5_06_H
E029	TWP_190	35.25158	-85.74639	NheI_A	EcoRI_05	iTru7_08_09	iTru5_05_A
E030	TWP_191	35.25158	-85.74639	NheI_B	EcoRI_06	iTru7_08_10	iTru5_05_B
E031	TWP_193	34.975928	-85.240603	NheI_C	EcoRI_07	iTru7_08_11	iTru5_05_C
E032	TWP_194	35.950669	-83.876807	NheI_D	EcoRI_08	iTru7_08_12	iTru5_05_D
E033	TWP_203	35.18727	-84.48791	NheI_E	EcoRI_09	iTru7_09_01	iTru5_05_E
E034	TWP_233	35.74452	-82.214	NheI_F	EcoRI_10	iTru7_09_02	iTru5_05_F
E035	TWP_238	34.67045	-85.06178	NheI_G	EcoRI_11	iTru7_09_03	iTru5_05_G
E036	TWP_242	34.21607	-84.68336	NheI_H	EcoRI_12	iTru7_09_04	iTru5_05_H
E037	TWP_247	35.19822	-84.37076	NheI_A	EcoRI_01	iTru7_09_05	iTru5_04_A
E038	TWP_248	35.19362	-84.38221	NheI_B	EcoRI_02	iTru7_09_06	iTru5_04_B
E039	TWP_255	31.18654	-89.13753	NheI_C	EcoRI_03	iTru7_09_07	iTru5_04_C
E040	TWP_254	33.25106	-88.98165	NheI_D	EcoRI_04	iTru7_09_08	iTru5_04_D
E041	TWP_259	33.428306	-84.946667	NheI_E	EcoRI_05	iTru7_09_09	iTru5_04_E
E042	TWP_260	36.781152	-80.379511	NheI_F	EcoRI_06	iTru7_09_10	iTru5_04_F
E043	TWP_271	34.785125	-83.933586	NheI_B	EcoRI_10	iTru7_10_02	iTru5_03_B
E044	TWP_273	36.25015	-83.92902	NheI_C	EcoRI_11	iTru7_10_03	iTru5_03_C
E045	KHK_615	36.288	-81.6492	NheI_D	EcoRI_12	iTru7_10_04	iTru5_03_D
E046	KHK_595	35.8503	-81.9855	NheI_E	EcoRI_01	iTru7_10_05	iTru5_03_E
E047	KHK_596	35.8503	-81.9855	NheI_F	EcoRI_02	iTru7_10_06	iTru5_03_F

Table S1. Continued.

Sample Name	Catalog Number	Latitude	Longitude	Left Adapter	Right Adapter	iTru7	iTru5
E048	KHK_448	35.1408	-81.3458	NheI_G	EcoRI_03	iTru7_10_07	iTru5_03_G
E049	KHK_727	35.7813	-85.0198	NheI_H	EcoRI_04	iTru7_10_08	iTru5_03_H
E050	199	31.0422	-89.0717	NheI_A	EcoRI_01	iTru7_10_09	iTru7_10_D
E051	373	36.2737	-86.9028	NheI_B	EcoRI_02	iTru7_10_10	iTru7_10_E
E052	KHK688	35.6253	-86.3165	NheI_C	EcoRI_03	iTru7_10_11	iTru7_10_F
E053	KHK402	36.8468	-85.2118	NheI_D	EcoRI_04	iTru7_10_12	iTru7_10_G
E054	8.331	34.08	-82.34	NheI_E	EcoRI_05	iTru7_11_01	iTru7_10_H
E055	KHK469	35.8217	-78.7883	NheI_F	EcoRI_06	iTru7_11_02	iTru5_09_A
E056	KHK378	37.083782	-87.031199	NheI_G	EcoRI_07	iTru7_11_03	iTru5_09_B
E057	KHK416	37.7825	-83.6732	NheI_H	EcoRI_08	iTru7_11_04	iTru5_09_C
E058	426	36.0703	-84.5447	NheI_A	EcoRI_09	iTru7_11_05	iTru5_09_D
E059	8.17	30.33	-84.5	NheI_B	EcoRI_10	iTru7_11_06	iTru5_09_E
E060	186	31.0957	-89.2138	NheI_C	EcoRI_11	iTru7_11_07	iTru5_09_F
E061	8.98	35.1	-83.68	NheI_D	EcoRI_12	iTru7_11_08	iTru5_09_G
E062	735	36.2737	-86.9028	NheI_E	EcoRI_01	iTru7_11_09	iTru5_09_H
E063	374	37.0558	-87.6442	NheI_F	EcoRI_02	iTru7_11_10	iTru5_08_A
E064	506	35.9647	-81.3145	NheI_G	EcoRI_03	iTru7_11_11	iTru5_08_B
E065	159	39	-84.72	NheI_H	EcoRI_04	iTru7_11_12	iTru5_08_C
E066	71203	37.99053	-82.350224	NheI_A	EcoRI_05	iTru7_12_01	iTru5_08_D
E067	562	38.924183	-78.331312	NheI_B	EcoRI_06	iTru7_12_02	iTru5_08_E
E068	730	36.001299	-84.508564	NheI_C	EcoRI_07	iTru7_12_03	iTru5_08_F
E069	H-1915	34.5875	-88.1917	NheI_D	EcoRI_08	iTru7_12_04	iTru5_08_G
E070	KHK391	37.212141	-86.137876	NheI_E	EcoRI_09	iTru7_12_05	iTru5_08_H
E071	TWP_106	35.68378	-83.53802	NheI_A	EcoRI_01	iTru7_12_06	iTru5_12_D
E072	TWP_325	36.23755	-84.11978	NheI_B	EcoRI_02	iTru7_12_07	iTru5_12_E
E073	TWP_319	35.184663	-85.868269	NheI_C	EcoRI_03	iTru7_12_08	iTru5_12_F
E074	TWP_051	34.95406	-83.5527	NheI_D	EcoRI_04	iTru7_12_09	iTru5_12_G
E075	TWP_084	33.890974	-83.362278	NheI_E	EcoRI_05	iTru7_12_10	iTru5_12_H

Table S1. Continued.

Sample Name	Catalog Number	Latitude	Longitude	Left Adapter	Right Adapter	iTru7	iTru5
E076	TWP_295	35.77439	-83.11215	NheI_F	EcoRI_06	iTru7_12_11	iTru5_11_A
E077	TWP_299	35.96337	-82.8667	NheI_G	EcoRI_07	iTru7_12_12	iTru5_11_B
E078	TWP_300	35.96337	-82.8667	NheI_H	EcoRI_08	iTru7_13_01	iTru5_11_C
E079	TWP_196	36.03712	-81.90831	NheI_A	EcoRI_09	iTru7_13_02	iTru5_11_D
E080	TWP_304	35.357547	-83.917827	NheI_B	EcoRI_10	iTru7_13_03	iTru5_11_E
E081	TWP_219	35.99546	-84.47674	NheI_C	EcoRI_11	iTru7_13_04	iTru5_11_F
E082	TWP_195	36.03712	-81.90831	NheI_D	EcoRI_12	iTru7_13_05	iTru5_11_G
E083	TWP_309	35.741028	-83.074309	NheI_E	EcoRI_01	iTru7_13_06	iTru5_11_H
E084	TWP_311	35.783171	-83.116172	NheI_F	EcoRI_02	iTru7_13_07	iTru5_10_A
E085	TWP_306	35.7445	-83.00855	NheI_G	EcoRI_03	iTru7_13_08	iTru5_10_B
E086	TWP_307	35.783171	-83.116172	NheI_H	EcoRI_04	iTru7_13_09	iTru5_10_C
E087	KHK492	36.3932	-80.2693	NheI_A	EcoRI_01	iTru7_13_10	iTru5_01_A
E088	KHK525	37.8777	-79.8097	NheI_B	EcoRI_02	iTru7_13_11	iTru5_01_B
E089	485	36.4825	-77.9677	NheI_C	EcoRI_03	iTru7_13_12	iTru5_01_C
E090	KHK49	34.728	-84.378	NheI_E	EcoRI_05	iTru7_06_02	iTru5_01_E
E091	KHK397	37.0838	-87.0312	NheI_F	EcoRI_06	iTru7_06_03	iTru5_01_F
E092	KHK536	40.68	-74.38	NheI_G	EcoRI_07	iTru7_06_04	iTru5_01_G
E093	50sp#1	34.728	-84.378	NheI_H	EcoRI_08	iTru7_06_05	iTru5_01_H
E094	7.104sp#1	35.05	-83.19	NheI_A	EcoRI_09	iTru7_06_06	iTru5_02_A
E095	199sp#1	31.0422	-89.0717	NheI_B	EcoRI_10	iTru7_06_07	iTru5_02_B
E096	5.28	35.35	-83.91	NheI_C	EcoRI_11	iTru7_06_08	iTru5_02_C
E097	KHK607	36.038	-81.91	NheI_D	EcoRI_12	iTru7_06_09	iTru5_02_D
E098	KHK602	36.038	-81.91	NheI_E	EcoRI_01	iTru7_06_10	iTru5_02_E
E099	KHK420	37.801	-83.767	NheI_F	EcoRI_02	iTru7_06_11	iTru5_02_F
E100	KHK574	38.4662	-78.5017	NheI_G	EcoRI_03	iTru7_06_12	iTru5_02_G
E101	KHK261	35.3775	-87.5117	NheI_H	EcoRI_04	iTru7_07_01	iTru5_02_H
E102	KHK605	36.038	-81.91	NheI_A	EcoRI_05	iTru7_07_02	iTru5_03_A
E103	KHK466	35.31	-79.25	NheI_B	EcoRI_06	iTru7_07_03	iTru5_03_B

Table S1. Continued.

Sample Name	Catalog Number	Latitude	Longitude	Left Adapter	Right Adapter	iTru7	iTru5
E104	KHK246	35.5998	-87.7727	NheI_C	EcoRI_07	iTru7_07_04	iTru5_03_C
E105	TWP_077	34.89997	-84.97665	NheI_E	EcoRI_09	iTru7_07_06	iTru5_03_E
E106	TWP_109	36.71878	-81.52039	NheI_C	EcoRI_03	iTru7_07_08	iTru5_03_G
E107	TWP_111	36.64	-81.58842	NheI_D	EcoRI_04	iTru7_07_09	iTru5_03_H
E108	TWP_112	35.78635	-85.01464	NheI_E	EcoRI_05	iTru7_07_10	iTru5_04_A
E109	TWP_114			NheI_F	EcoRI_06	iTru7_07_11	iTru7_04_B
E110	TWP_129	36.07477	-84.542112	NheI_G	EcoRI_07	iTru7_07_12	iTru5_04_C
E111	TWP_124	30.50223	-84.21562	NheI_H	EcoRI_08	iTru7_08_01	iTru5_04_D
E112	TWP_031	34.94785	-83.55234	NheI_A	EcoRI_09	iTru7_08_02	iTru5_04_E
E113	TWP_046			NheI_B	EcoRI_10	iTru7_08_03	iTru7_04_F
E114	TWP_061			NheI_C	EcoRI_11	iTru7_08_04	iTru7_04_G
E115	TWP_263	37.378127	-80.244161	NheI_A	EcoRI_01	iTru7_13_09	iTru5_10_E
E116	TWP_269	34.0138	-87.3583	NheI_B	EcoRI_02	iTru7_13_10	iTru5_10_F
E117	TWP_270	33.88312	-86.58096	NheI_C	EcoRI_03	iTru7_06_03	iTru5_01_C
E118	53644	35.312992	-79.261606	NheI_E	EcoRI_05	iTru7_08_09	iTru5_02_D
E119	53647	35.312992	-79.261606	NheI_D	EcoRI_06	iTru7_08_10	iTru5_02_E
E120	DB_9660	35.02803	-79.63772	NheI_C	EcoRI_07	iTru7_08_11	iTru5_02_F
E121	TWP_703	30.942	-89.9783	NheI_A	EcoRI_01	iTru7_10_01	iTru7_11_A
E122	H-1873	30.83	-90.2	NheI_B	EcoRI_02	iTru7_10_02	iTru7_11_B
E123	H-19874	30.83	-90.2	NheI_C	EcoRI_03	iTru7_10_03	iTru7_11_C
E124	H-532	30.83	-90.2	NheI_D	EcoRI_04	iTru7_10_04	iTru7_11_D

Table S2: `Structure` results from replicate contact zones. Results are shown only for admixture models. Samples from Sites 1–4 have sample names beginning with A–D, respectively.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
A001	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A002	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A003	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A004	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A005	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A006	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A007	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A008	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A009	1.00	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00
A010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A011	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A013	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A014	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A015	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A016	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A017	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A018	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A019	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A020	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A021	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A022	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A023	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A024	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A025	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A026	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	Posterior Mean Estimates of Admixture Proportions											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
A027	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A028	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A029	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A030	1.00	1.00	1.00	1.00	1.00	0.99	0.99	0.97	0.98	0.98	1.00	1.00
B001	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B002	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B003	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B004	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B005	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B006	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B007	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B008	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B009	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B011	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B012	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B013	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B014	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B015	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B016	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B017	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B018	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B020	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B021	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B022	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
B023	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B024	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B025	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B026	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B027	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B028	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B029	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B030	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B031	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B032	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B033	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B034	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B035	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B036	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B037	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B038	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B039	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B040	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B041	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B042	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B043	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B044	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B045	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B046	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B047	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B048	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	Posterior Mean Estimates of Admixture Proportions											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
B049	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B050	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B051	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B052	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B053	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B054	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B055	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B056	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B057	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B058	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B059	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B060	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B061	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B062	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B063	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B064	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B065	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B066	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B067	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B068	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B069	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B070	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B071	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B072	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B073	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B074	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
B075	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B076	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B077	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B078	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B079	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B080	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B081	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B082	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B083	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B084	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B085	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B086	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B087	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B088	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B089	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B090	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B091	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B092	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B093	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B094	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B095	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B096	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B097	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B098	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00
B099	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
C001	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00
C002	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C003	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C004	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C005	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C006	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C007	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C008	0.99	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C009	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C010	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C011	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C013	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C014	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C015	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C016	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C017	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C018	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C019	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C020	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C021	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C022	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C023	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C024	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C025	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C026	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
C027	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C028	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C029	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C030	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C031	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C032	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C033	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C034	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C035	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C036	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C037	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C038	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C039	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C040	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C041	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C042	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C043	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C044	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C045	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C046	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.98	0.97
C047	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C048	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C049	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C050	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C051	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C052	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
C053	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C054	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C055	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C056	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C057	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C058	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C059	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C060	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C061	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00
C062	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C063	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C064	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C065	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C066	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C067	1.00	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C068	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C069	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C070	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C071	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C072	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C073	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C074	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C075	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C076	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.99	1.00	0.99
C077	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C078	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
C079	0.98	1.00	0.96	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C080	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C081	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C082	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C083	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C084	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C085	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C086	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C087	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C088	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
C089	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C090	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C091	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C092	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C093	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C094	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C095	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C096	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C097	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C098	0.99	1.00	0.99	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C099	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D001	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D002	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D003	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D004	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
D005	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D006	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D007	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D008	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D009	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D011	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D012	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D013	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D014	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D015	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D016	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D017	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D018	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D020	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D021	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D022	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D023	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D024	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D025	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D026	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D027	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D028	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D029	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D030	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S2. Continued.

Sample	Posterior Mean Estimates of Admixture Proportions											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
D031	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D032	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D033	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D034	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D035	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D036	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D037	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D038	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00
D039	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D040	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D041	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D042	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D043	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D044	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D045	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D046	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D047	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D048	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D049	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D050	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D051	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D052	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D053	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D054	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D055	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D056	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
D057	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D058	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D059	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D060	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D061	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D062	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D063	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D064	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D065	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D066	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D067	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D068	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01
D069	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D070	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D071	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D072	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D073	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D074	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D075	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D076	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01
D077	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D078	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D079	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D080	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D081	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D082	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S2. Continued.

Sample	**Posterior Mean Estimates of Admixture Proportions**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
D083	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D084	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D085	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D086	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D087	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D088	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D089	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D090	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D091	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D092	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D093	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D094	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D095	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D096	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D097	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D098	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D099	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3: `DAPC` results from replicate contact zones. Samples from Sites 1–4 have sample names beginning with A–D, respectively.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
A001	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A002	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A003	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A004	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A005	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A006	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A007	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A008	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A009	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A011	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A013	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A014	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A015	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A016	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A017	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A018	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A019	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A020	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A021	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A022	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A023	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A024	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A025	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A026	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
A027	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A028	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A029	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
A030	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B001	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B002	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B003	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B004	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B005	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B006	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B007	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B008	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B009	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B011	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B012	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B013	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B014	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B015	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B016	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B017	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B018	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B020	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B021	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B022	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
B023	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B024	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B025	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B026	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B027	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B028	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B029	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B030	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B031	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B032	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B033	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B034	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B035	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B036	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B037	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B038	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B039	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B040	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B041	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B042	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B043	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B044	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B045	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B046	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B047	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B048	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
B049	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B050	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B051	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B052	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B053	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B054	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B055	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B056	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B057	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B058	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B059	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B060	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B061	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B062	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B063	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B064	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B065	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B066	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B067	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B068	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B069	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B070	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B071	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B072	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B073	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B074	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
B075	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B076	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B077	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B078	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B079	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B080	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B081	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B082	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B083	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B084	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B085	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B086	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B087	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B088	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B089	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B090	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B091	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B092	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B093	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B094	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
B095	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B096	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B097	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B098	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B099	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
B100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
C001	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C002	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C003	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C004	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C005	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C006	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C007	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C008	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C009	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C010	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C011	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C013	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C014	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C015	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C016	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C017	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C018	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C019	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C020	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C021	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C022	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C023	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C024	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C025	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C026	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
C027	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C028	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C029	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C030	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C031	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C032	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C033	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C034	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C035	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C036	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C037	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C038	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C039	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C040	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C041	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C042	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C043	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C044	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C045	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C046	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C047	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C048	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C049	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C050	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C051	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C052	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
C053	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C054	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C055	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C056	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C057	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C058	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C059	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C060	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C061	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C062	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C063	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C064	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C065	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C066	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C067	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C068	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C069	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C070	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C071	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C072	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C073	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C074	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C075	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C076	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C077	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C078	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
C079	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C080	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C081	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C082	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C083	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C084	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C085	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C086	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C087	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C088	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C089	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C090	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C091	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C092	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C093	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C094	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C095	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C096	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C097	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C098	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C099	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D001	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D002	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D003	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D004	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
D005	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D006	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D007	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D008	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D009	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D011	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D012	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D013	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D014	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D015	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D016	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D017	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D018	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D019	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D020	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D021	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D022	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D023	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D024	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D025	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D026	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D027	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D028	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D029	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D030	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
D031	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D032	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D033	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D034	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D035	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D036	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D037	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D038	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D039	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D040	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D041	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D042	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D043	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D044	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D045	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D046	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D047	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D048	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D049	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D050	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D051	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D052	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D053	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D054	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D055	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D056	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
D057	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D058	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D059	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D060	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D061	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D062	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D063	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D064	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D065	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D066	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D067	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D068	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D069	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D070	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D071	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D072	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D073	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D074	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D075	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D076	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D077	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D078	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D079	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D080	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D081	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D082	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Table S3. Continued.

Sample	**Posterior Membership Probabilities**											
	min5				*min100*				*min250*			
	all SNPs		one SNP		all SNPs		one SNP		all SNPs		one SNP	
	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05	-	>0.05
D083	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D084	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D085	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D086	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D087	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D088	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D089	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D090	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D091	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D092	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D093	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D094	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D095	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D096	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D097	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D098	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D099	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D100	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

D Supplemental Figures

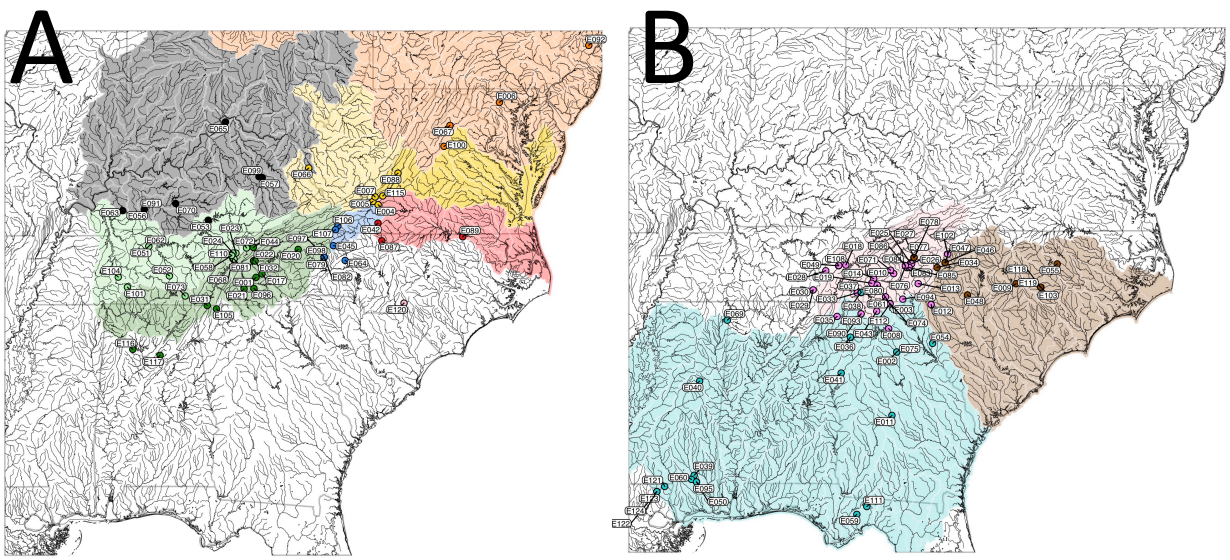

Figure S1: Maps of sampling localities (including sample labels), with colors corresponding to major mitochondrial and genomic lineages and corresponding river drainages. A) "northern" clade; B) "southern" clade. Black = Muskingum, Scioto, Great Miami, Middle Ohio, Kentucky-Licking, Green, Wabash, and Ohio River drainages; Light green = Cumberland, Middle Tennessee-Elk, and Tennessee drainages; Dark green = Upper Tennessee, Middle Tennessee-Hiwassee, and Middle Tennessee-Elk River drainages; Orange = Upper Hudson, Hudson-Long Island, Delaware-Mid Atlantic Coastal, Susquehanna, Upper Chesapeake, Potomac, Western Lake Erie, Southern Lake Erie, Allegheny, Monongahela, and portions of the Upper Ohio River drainages; Light yellow = Big Sandy-Guyandotte, portions of the Upper Ohio, and portions of the Kanawha River drainage; Dark yellow = Chesapeake and James River drainages; Royal blue = Upper New River drainage; Red = Chowan-Roanoke River drainage; Pink = Upper Tennessee and Middle Tennessee-Hiwassee River drainages; Brown = Neuse-Pamlico, Cape Fear, Pee Dee, and Edisto-Santee River drainages; Turquoise = Ogeechee-Savannah, Altamaha-St. Marys, Suwannee, Ochlockonee, Apalachicola, Choctawhatchee-Escambia, Alabama, Mobile-Tombigbee, Pascagoula, Pearl, Mississippi-Yazoo, and Mississippi-Big Black River drainages.

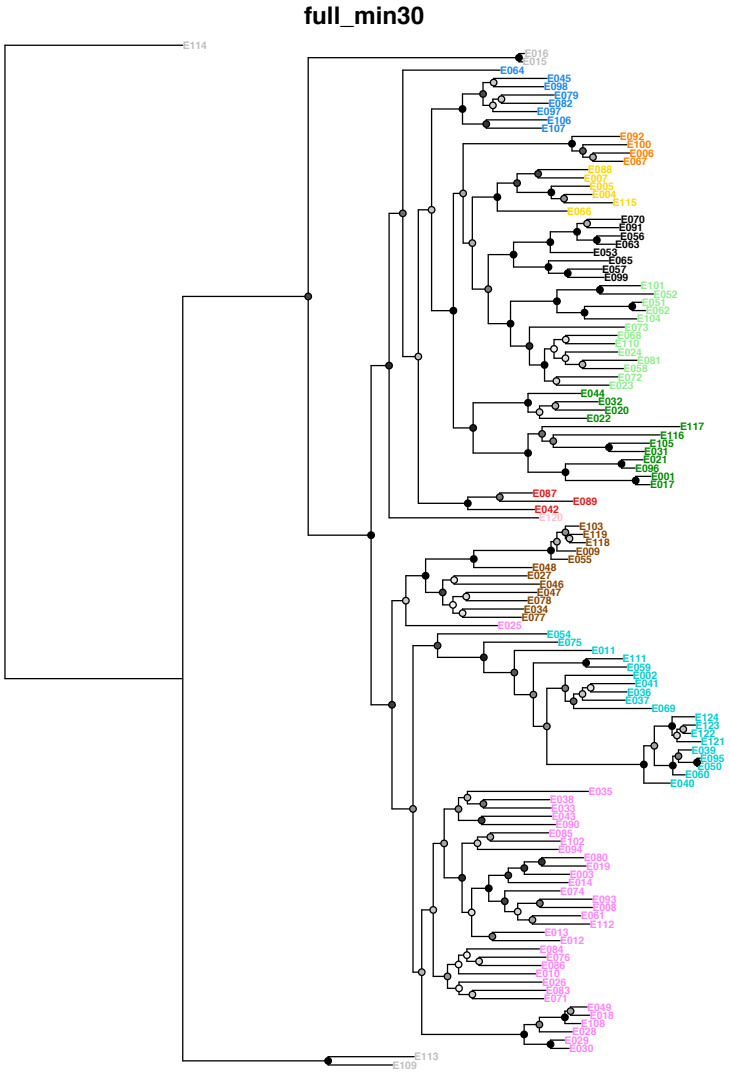

Figure S2: Maximum-likelihood phylogeny from *full_min30* assembly. Lineage labels and colors correspond to major mtDNA lineage names from Kozak et al. [106] and explained in the Methods, and continuous variation in node color represents bootstrap edge support from 0 (white) to 100 (black).

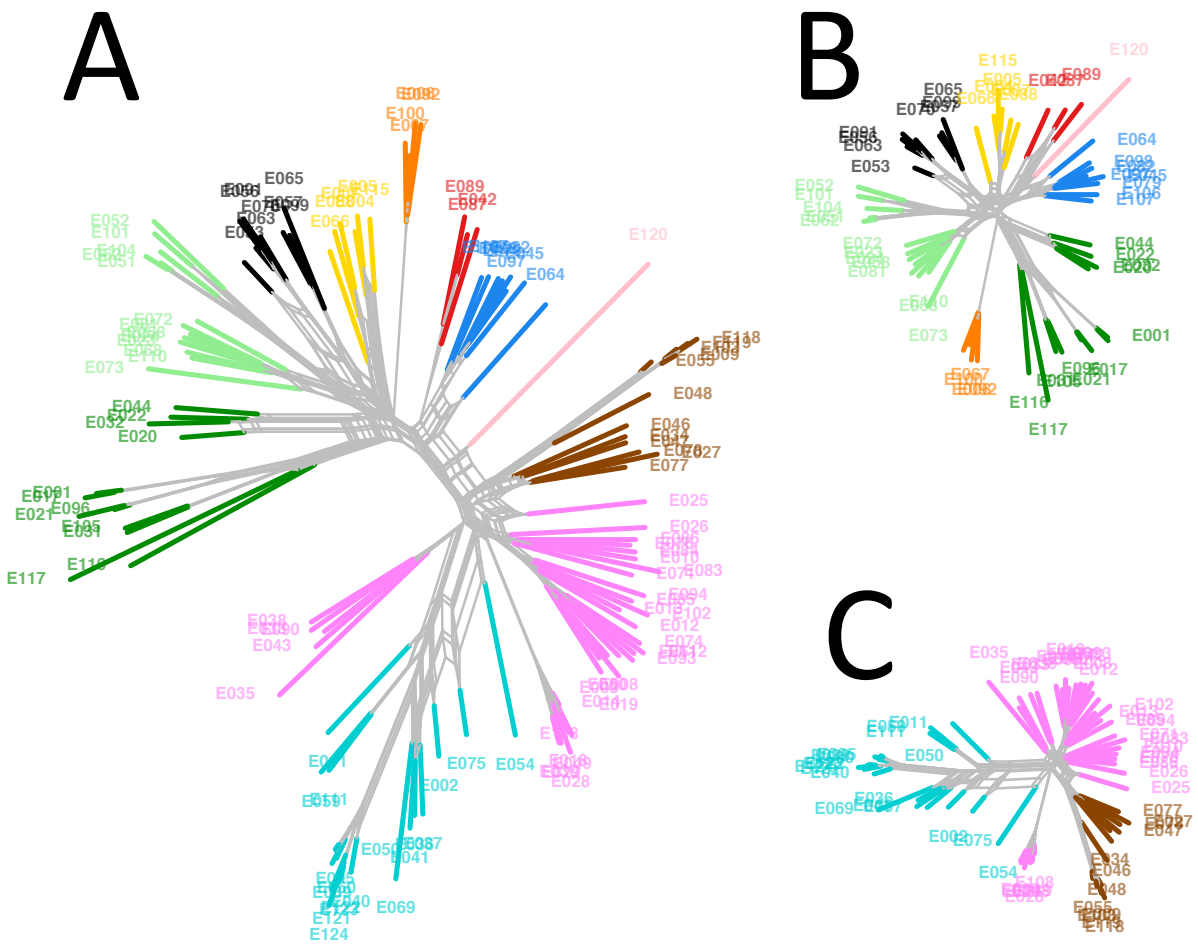

Figure S3: Distance-based phylogenetic networks (with sample labels) estimated from the *full_min5* assembly. A) all samples; B) only samples from the northern clade; and C) only samples from the southern clade. Colors correspond to major lineages as explained in the Results.

.

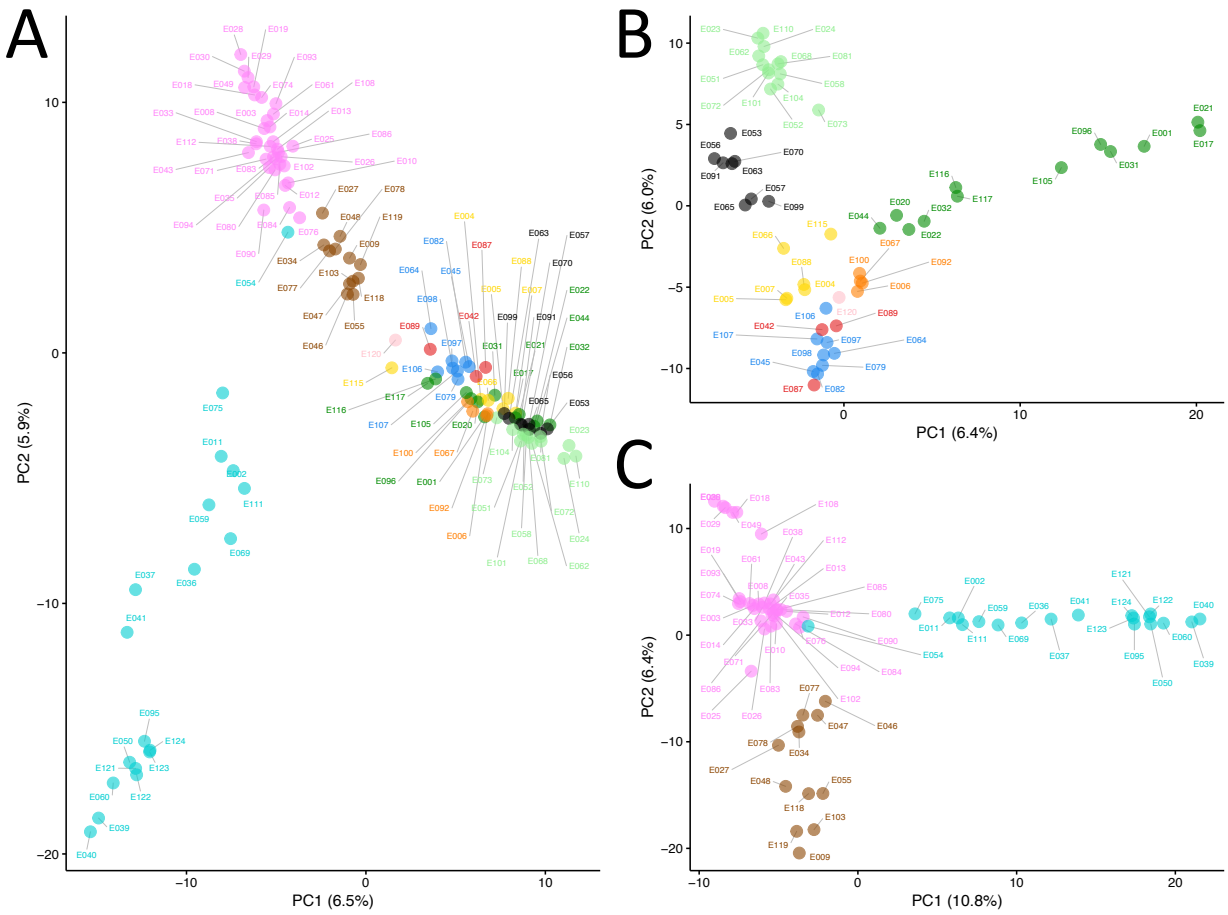

Figure S4: Principal components analyses (with sample labels) from the full_min5 assembly. A) all samples and all SNPs; B) only samples from the northern clade; and C) only samples from the southern clade. Colors correspond to major lineages as explained in the Results.

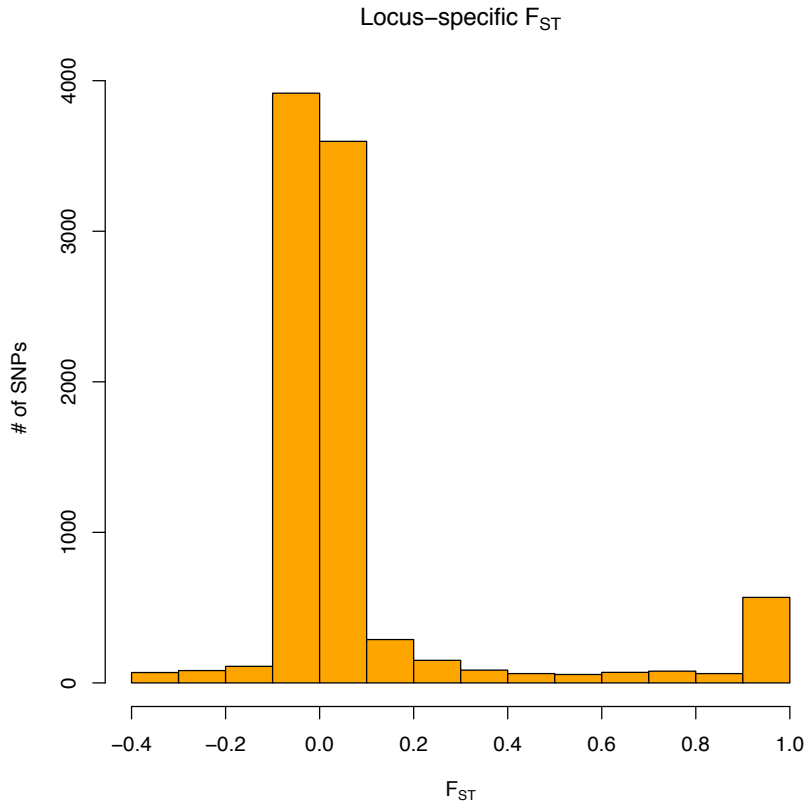

Figure S5: Distribution of locus-specific F_{ST} from the *min5* RADcap assembly

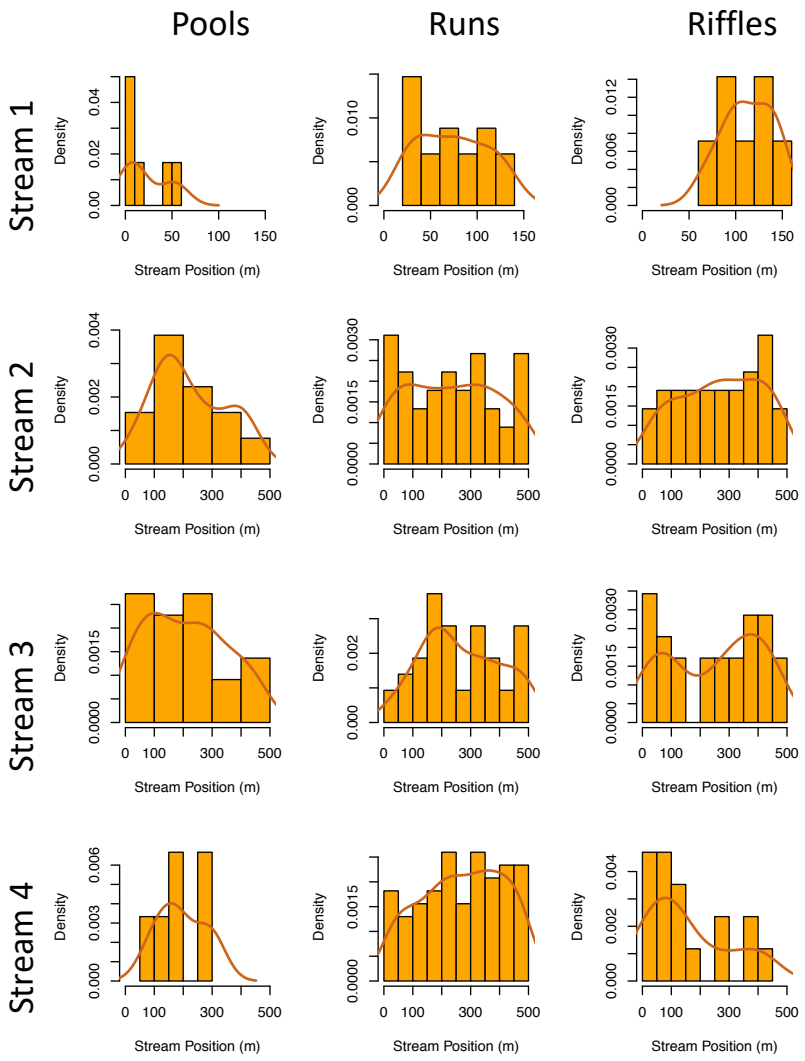

Figure S6: Frequency histograms and kernel density plots of the linear position of stream habitats across each of four replicate contact zones.

Vita

Todd W. Pierson is originally from Indianapolis, Indiana and graduated with a B.S. Ecology from the University of Georgia in 2013. He uses genomic, behavioral, and field observational data to study the ecology, evolution, and conservation of amphibians. More information about his research is available at his personal website www.twpierson.com.