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A Stable Niche Assumption-free Test of Ecological Divergence

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A stable niche assumption-free test of ecological divergence

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ABSTRACT

Understanding the impact of geological events on diversification processes is central to evolutionary ecology. The recent amalgamation between ecological niche models (ENMs) and phylogenetic analyses has been used to estimate historical ranges of modern lineages by projecting current ecological niches of organisms onto paleoclimatic reconstructions. A critical assumption underlying this approach is that niches are stable over time. Using *Notophthalmus viridescens* (eastern newt), in which four ecologically diverged subspecies are recognized, we introduce an analytical framework free from the niche stability assumption to examine how refugial retreat and subsequent postglacial expansion have affected intraspecific ecological divergence. We found that the current subspecies designation was not congruent with the phylogenetic lineages. Thus, we examined ecological niche overlap between the refugial and modern populations, in both subspecies and lineage, by creating ENMs independently for modern and estimated last glacial maximum (LGM) newt populations, extracting bioclimate variables by randomly generated points, and conducting principal component analyses. Our analyses consistently showed that when tested as a hypothesis, rather than used as an assumption, the niches of *N. viridescens* lineages have been unstable since the LGM (both subspecies and lineages). There was greater ecological niche differentiation among the subspecies than the modern phylogenetic lineages, suggesting that the subspecies, rather than the phylogenetic lineages, is the unit of the current ecological divergence. The present study found little evidence that the LGM refugial retreat caused the currently observed ecological divergence and suggests that ecological divergence has occurred during postglacial expansion to the current distribution ranges.

1. Introduction

Geographic range contraction, fragmentation, and expansion caused by Pleistocene glacial cycles all have had a major impact on the spatial distribution of genetic variation across many taxa (Avise et al., 1998; Hewitt, 2000; Zamudio and Savage, 2003; Steele and Storfer, 2006; Walker et al., 2009). The prominence of such geological events, particularly that of glacial retreat since the last glacial maximum (LGM), has led to questions regarding their influence on evolutionary diversification. Our ability to address the influence of geological processes on diversification has greatly improved with the recent development of methods that facilitate phylogenetic hypothesis testing coupled with the advent of ecological niche models (ENMs). For example, ENMs offer means to evaluate phylogeographic inferences based on simulated past distribution patterns (Knowles et al., 2007; Richards et al., 2007; Peterson and Nyari, 2008; Walker et al., 2009), as well as to test current niche divergence among closely related lineages (Graham et al., 2004; Rissler and Apodaca, 2007; Pyron and Burbrink, 2009).

Predicting past ENMs is an attractive and promising tool for the fields such as biogeography, evolutionary ecology, paleontology, phylogenetics, and systematics. However, the current method of developing historical ENMs relies on present-day species distribution data to predict past niches and thus rests on the assumption that ecological niches of target organisms are stable through time (Nogués-Bravo, 2009). This stable-niche assumption is problematic for at least three reasons. First, without abundant paleoecological

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and paleontological evidence, it is often difficult to corroborate the assumption of niche stability. Second, the increasing evidence for ecological speciation (Nosil et al., 2009; Schluter, 2009) suggests notable exceptions (but see Wiens, 2004; Wiens and Graham, 2005; Kozak and Wiens, 2010 for speciation via niche conservatism). Finally, there is no quantifiable definition of what constitutes a sufficiently "stable niche," wherein current niche variables can be appropriately used to predict past ENMs (Nogués-Bravo, 2009). As a consequence of the difficulty in assessing niche stability through time, there has been little attempt to test for an ecological niche shift between the past (e.g., LGM) and the present using ENMs. In response to environmental changes typical of large scale geologic events such as the LGM, populations must move, adapt, or suffer extinction.

Previous studies that integrate ENMs with phylogeographic analyses generate past ENMs based on the stable-niche assumption to evaluate inferences drawn from population genetic structures (Hugall et al., 2002; Carstens and Richards, 2007; Knowles et al., 2007; Waltari et al., 2007; Peterson and Nyari, 2008; Solomon et al., 2008; Jakob et al., 2009; Walker et al., 2009). While results from these disparate analyses are often in general agreement, Waltari et al. (2007) found that for 6 of 20 studied species, predicted historical distributions showed significant discordance between the predicted ENMs and phylogeographic analyses. Moreover, other studies found evidence of recent niche divergence between closely related taxa (Graham et al., 2004) and even within a single species (Rissler and Apodaca, 2007; Pyron and Burbrink, 2009). Furthermore, remarkably rapid niche shifts (less than 120 yrs) have been detected in a principal-component environmental space between the native and the invasive populations of spotted knapweed *Centaurea maculosa* (Broennimann et al., 2007) and Asian tiger mosquito *Aedes albopictus* (Medley, 2010). Indeed, there is increasing empirical evidence that adaptation to novel niches can occur rapidly, even potentially resulting in speciation events (see reviews in Rundle and Nosil, 2005; Nosil et al., 2009; Schluter, 2009). Thus, the assumption of stable ecological niches since the Pleistocene is not universal, and should rather be an empirical question. To this end, developing a framework for historical ENMs that are free from the stable-niche assumption would advance research that explores the importance of geological events in shaping ecological divergence of any species.

We used the North American eastern newt, *Notophthalmus viridescens*, as a model to introduce a niche-stability free analytical framework for examining the role of Pleistocene refugia in post-LGM ecological niche divergence. Many wide-ranging species exhibit considerable intraspecific phenotypic variation (e.g., elk [*Cervus canadensis*], greenish warblers [*Phylloscopus trochiloides*], northern cricket frog [*Acris crepitans*] and common kingsnake [*Lampropeltis getula*]). Such phenotypic variation often reflects local adaptation and thus offers opportunities for testing the effects of the LGM on intraspecific ecological divergence. *Notophthalmus viridescens* comprises four subspecies based on morphological variation and ecological differentiation (Mecham, 1967; Fig. 1). The results from the common garden experiments suggest that the ecological differences among the subspecies observed in nature are genetically-based, rather than plastic; the subspecies differ in the expression of the life history traits such as larval period, body mass at metamorphosis, and life cycle polyphenism (i.e., paedomorphic vs. metamorphic adults; Takahashi and Parris, 2008; Takahashi et al., 2011).

We focus on the LGM and the subsequent postglacial warming as a potential catalyst of ecological niche divergence in *N. viridescens*. Hypotheses for the timing of recent niche divergence include: (i) refugial retreat during the LGM, (ii) postglacial range expansion to novel environments following the LGM, or (iii) a combination of both. When fragmented populations that have retreated into distinct refugia experience different selective pressures and become adapted to different refugial environments, ecological divergence can occur in allopatry during the LGM. The pattern of ecological divergence attained during the LGM may still be maintained at present even after postglacial expansion reconnecting the fragmented populations (i.e., secondary contact; Schluter, 2001). In such a scenario, the current ecological niches are predicted to be similar to those during the LGM and we should observe phylogenetic clustering in niche space. Alternatively, postglacial range expansion following the LGM may allow organisms to reach novel habitats and may facilitate adaptive divergence (e.g., ecological speciation in postglacial fishes, Schluter, 1996). This scenario predicts the ecological disparity between the LGM and the current niches, suggesting the violation of the niche stability assumption.

To test the hypotheses pertaining to the ecological niche divergence of *N. viridescens*, we first tested phylogenetic predictions resulting from the assumption that their subspecies are congruent with phylogenetic lineages. We then adopt the statistical method of Lemmon and Lemmon (2008) to estimate the geographic location of ancestral populations in conjunction with paleoclimatic reconstructions to create LGM niche models for refugial lineages. Next, we quantitatively assess niche divergence by comparing contemporary to historical ENMs through principal component analyses and niche overlap analyses. Our test of phylogenetic concordance between refugial lineages and subspecies delineation establishes a critical foundation for the subsequent construction of the LGM ENMs and the niche comparison between the LGM populations and the current subspecies. Additionally, we tested for niche shift between the refugial populations and the contemporary phylogenetic lineages of *N. viridescens* when the current subspecies designation was not congruent with refugial lineages.

2. Materials and methods

2.1. Study organism

The subspecific patterns of life-history variation within *N. viridescens* are associated with the environmental conditions of the subspecies distributional ranges (Takahashi and Parris, 2008; Takahashi et al., 2011). Larvae of the most terrestrial subspecies, *N. v. viridescens* (the red-spotted newt), typically metamorphose rapidly to leave ponds as terrestrial juveniles (called efts) and return to aquatic habitats as adults in 3–7 years. This subspecies, which has two rows of distinct red spots on its dorsum, is predominantly distributed within the Appalachian Highlands (Fig. 1) where permanent wetlands are scarce, but ephemeral vernal pools are common (Babbitt and Groat, 1998). These areas also offer ideal woodland habitats for terrestrial salamanders as evidenced in the abundance and diversity of terrestrial plethodontid salamanders (Wilbur and Collins, 1973; Petranka, 1998). In contrast, *N. v. piaropicola* (the peninsula newt), which lacks any dorsal patternings, typically completes its entire life cycle in aquatic habitats. This almost exclusively aquatic subspecies is distributed in the Florida peninsula (Fig. 1) where relatively permanent wetlands are abundantly available. The sandy and hot terrestrial environment of Coastal Plain in Florida likely offers unsuitable habitats for terrestrial salamanders. *Notophthalmus v. dorsalis* (the broken-striped newt) with discontinuous two red lines on its dorsum and *N. v. louisianensis* (the central newt) with small inconspicuous red spots on its dorsum are distributed along the Interior and Coastal Plains (Fig. 1) where wetlands are relatively abundant, but have widely varying hydroperiods. These two subspecies have life-history characteristics intermediate between *N. v. viridescens* and *N. v. piaropicola*. Both subspecies exhibit life-cycle polyphenism (i.e.,

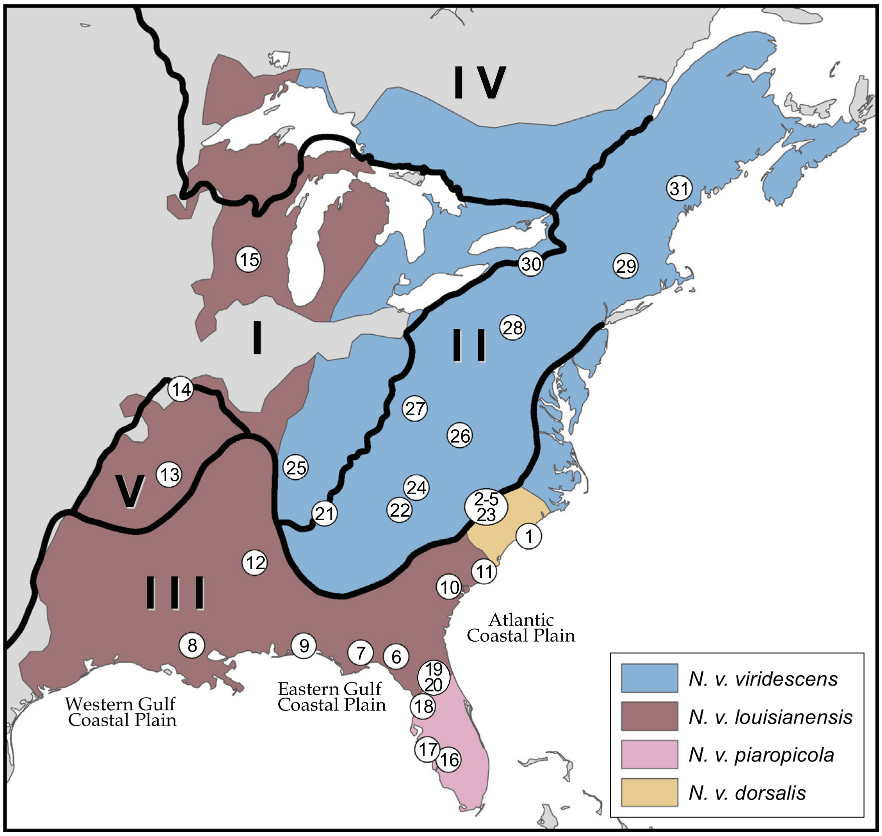


Fig. 1. *Notophthalmus viridescens* subspecies ranges (Mecham, 1967), physiographic natural regions, and sampling sites for the phylogeographic analyses. Physiographic natural regions are delineated by heavy, solid lines and identified as follows: I = Interior Plains, II = Appalachian Highlands, III = Atlantic Coastal Plain, IV = Laurentian Upland, and V = Ozark Plateaus.

developmental plasticity) in which larvae are able to metamorphose to terrestrial efts to leave drying aquatic habitats or, to remain in the water and directly mature into adults in ponds with long hydroperiods.

2.2. Tissue samples, DNA isolation, amplification, and sequencing

Tail clips from adults or terrestrial juveniles of *N. viridescens* were collected throughout its geographic range between 2004 and 2007 (Fig. 1, Table 1). Field-collected tissue samples were preserved in 95% ethanol and stored at -5 °C in the laboratory. As an outgroup, we included one specimen of *Taricha granulosa*, the most closely related genus to *Notophthalmus* (Weisrock et al., 2006). Divergence between *Taricha* and *Notophthalmus* was assumed to be roughly 35 Mya, following Zhang et al. (2008), and diversification of crown-group *Notophthalmus* was assumed to occur ca. 11 Mya (Zhang et al., 2008).

Genomic DNA was extracted using DNeasy tissue kit (Qiagen, Valencia, CA). A segment of roughly 1585 bases of mitochondrial DNA from part of the cytochrome *b* (cyt *b*) gene, part of subunit two of NADH dehydrogenase (ND2) gene, tRNA^{Ala}, tRNA^{Asn}, and tRNA^{Trp} were amplified by polymerase chain reaction (PCR). We amplified and cycle-sequenced *cyt b* using two primer combinations that we designed for this study, Noto1 (5′TGACCTACCAACACC ATCAAAT-3′)-Noto2 (5′GTATTTGG TGTTAATAGGGA AATT-3′) and NotoSeq1 (5′-TTACACAAATTATTACGGCCTA-3′)-NotoSeq2 (5′-TATGGGGTGAAATGCAATTTTGT-3′), respectively. We amplified and cycle-sequenced the other fragment consisting of tRNA^{Asn}, tRNA^{Ala}, tRNA^{Trp}, and ND2 using two primer combinations, L4437-H5934 and L4882a-H5692 following Weisrock et al. (2001). Amplification of *cyt b* was conducted with denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, sequence extension at 72 °C for 1 min per cycle for 30 cycles. Amplification of the continuous fragment of tRNA^{Asn}, tRNA^{Ala}, tRNA^{Trp}, and ND2 was conducted following the methods in Weisrock et al. (2001). PCR products were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA), cycle-sequenced using BigDye Terminator (Life Technologies, Carlsbad, CA), and sequenced with an Applied Biosystems 3730 automated sequencer. Cycle sequence reactions were run for both fragments with denaturation at 96 °C for 10 s, annealing at 50 °C for 10 s, sequence extension at 60 °C for 4 min per cycle for 35 cycles.

2.3. Pleistocene refugia, topological phylogeography and subspecies analyses

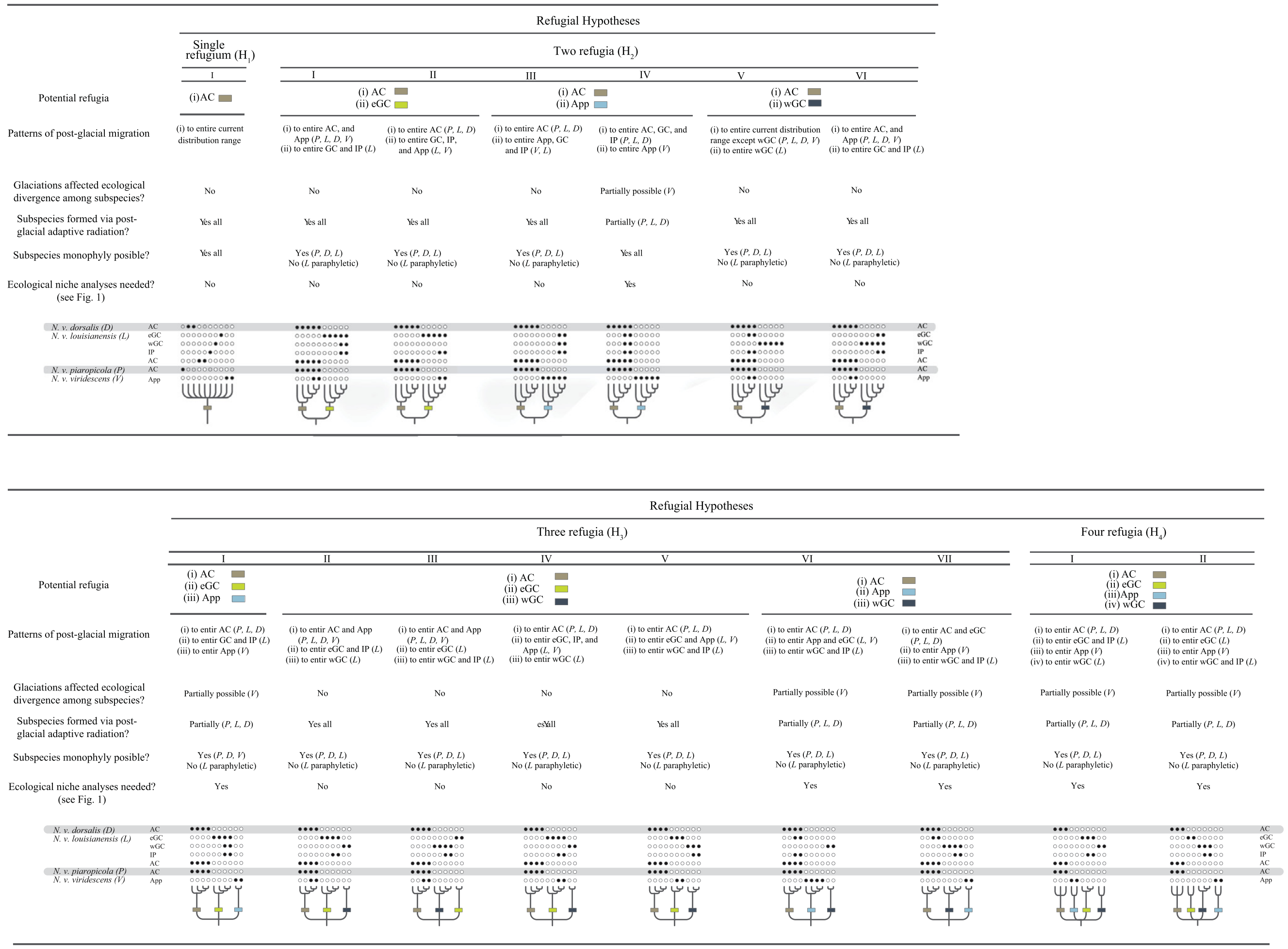
We generated 16 phylogeographically realistic predictions for the eastern newt based on the number and coarsely-defined location of Pleistocene refugia and pattern of the postglacial expansion (Fig. 2). We identified up to four possible refugia in the eastern United States based on previous studies. An Atlantic – Gulf genetic discontinuity is commonly observed in various taxa (as in freshwater fish: Burgess and Yerger, 1986; amphibians: Church et al., 2003; Pauly et al., 2007; reptiles: Walker and Avise, 1998; and mammals:Ellsworth et al., 1994; also see review in Soltis et al., 2006) presumably because organisms were fragmented and retreated into an Atlantic or eastern Gulf Coast refugia during the LGM (Avise, 1992; Duellman, 1999; Church et al., 2003; Pauly et al., 2007). Postglacial warming then allowed organisms to extend their ranges (Pielou, 1991; Williams et al., 2000). At least two additional Pleistocene refugia in the eastern United States have been suggested: an Appalachian Highland refugium (Church et al., 2003; Zamudio and Savage, 2003; Soltis et al., 2006) and a

Table 1
Populations, subspecies designation, sample locality, sequenced sample size, and haplotypes of *N. viridescens* used in our phylogenetic analyses. Haplotypes are characterized as follows: A.C. sub = Atlantic Coastal subclade; eastern G.C. sub = eastern Gulf Coastal subclade; A.H. = Appalachian Highland clade; and western G.C. = Western Gulf Coastal clade (see Fig. 3).

Pop. no.	Sample ID	Subspecies	State	GPS (decimal) north; west	Haplotypes	No. sequenced	Collectors
1	ABL	*dorsalis*	NC	33.99; 77.99	A.C. sub 1–6	10	A. Braswell
2	GSP	*dorsalis*	NC	34.92; 79.57	A.C. sub 7–14	8	M.K. Takahashi, Y. Takahashi
3	BS	*dorsalis*	NC	34.97; 79.35	A.C. sub 6, 13, 15–19	11	M.K. Takahashi
4	BFT	*dorsalis*	NC	35.07; 79.63	A.C. sub 13, 20–27 A.H. 1	31	T. Sharp, M.K. Takahashi
5	CFP	*dorsalis*	NC	35.08; 79.58	A.C. sub 13, 14, 20, 27, 29, 30 A.H. 15	10	M.K. Takahashi
6	MDSN	*louisianensis*	FL	30.26; 83.31	Eastern G.C. sub 11–15	8	P. Moler
7	LBTFL	*louisianensis*	FL	30.39; 84.66	Eastern G.C. sub 11	2	P. Moler
8	TAK	*louisianensis*	LA	30.48; 91.03	Western G.C. 1–2	10	T. Ohya, M.K. Takahashi
9	SRFL	*louisianensis*	FL	30.62; 86.80	Eastern G.C. sub 30	4	P. Moler
10	TSR	*louisianensis*	SC	32.48; 81.20	A.C. sub 31–38	10	M.K. Takahashi
11	FSPD	*louisianensis*	SC	32.93; 79.82	A.C. sub 39–42	4	M.K. Takahashi
12	ALT	*louisianensis*	MS	33.28; 88.78	Eastern G.C. sub 1–10	10	R. Altig
13	ARP	*louisianensis*	AR	35.97; 92.40	Eastern G.C. sub 16,17 A.H. 41	10	M.K. Takahashi
14	MO	*louisianensis*	MO	38.75; 92.19	Eastern G.C. sub 18,19 A.H. 26	5	J. R. Johnson
15	RBH	*louisianensis*	WI	43.09; 89.61	Eastern G.C. sub 20,21	6	R. Hay
16	GLDFL	*piaropicola*	FL	26.83; 81.47	A.C. sub 46–47	2	P. Moler
17	SRST	*piaropicola*	FL	27.20; 82.23	A.C. sub 48–50	3	P. Moler
18	HRND	*piaropicola*	FL	28.62; 82.35	A.C. sub 51–52	2	P. Moler
19	ALCFL	*piaropicola*	FL	29.53; 82.03	A.C. sub 53–58	10	P. Moler, G. Clark
20	PLM	*piaropicola*	FL	29.54; 81.84	A.C. sub 59–62	4	P. Moler
21	ZFX	*viridescens*	AL	34.92; 86.08	A.H. 27–30	7	Z. Felix, M.K. Takahashi
22	JFH	*viridescens*	SC	35.03; 83.07	Eastern G.C. sub 22 Eastern G.C. sub 23–26 A.C. sub 43,44	9	J. Humphries, M.K. Takahashi
23	CKP	*viridescens*	NC	35.07; 79.78	A.H. 1–3, 16–20	8	Y. Takahashi, M.K. Takahashi
24	ASH	*viridescens*	NC	35.75; 82.38	Eastern G.C. sub 22, 27–29 A.C. sub 45 A.H. 21	7	J. Petranka, M.K. Takahashi
25	CLB	*viridescens*	TN	36.40; 87.27	A.H. 31–34	4	M.K. Takahashi
26	KLG	*viridescens*	VA	37.37; 80.52	A.H. 4, 22–25, 36–38	12	K. L. Grayson
27	WVT	*viridescens*	WV	38.30; 82.33	A.H. 39	7	A. Mann, M.K. Takahashi
28	TRL	*viridescens*	PA	40.76; 78.01	A.H. 5–9, 40	15	T. Raffe
29	STT	*viridescens*	MA	42.38; 72.80	A.H. 10	4	S.G. Tilley
30	SEM	*viridescens*	NY	42.77; 76.98	A.H. 9–11	7	S. Myers
31	CRB	*viridescens*	ME	44.63; 69.92	A.H. 10, 12–14	6	C.R. Bevier

Western Gulf Coast refugium (Swenson and Howard, 2005; Walker et al., 2009). All four refugia are within the current distributional range of *N. viridescens*. The possibility of broad geographic expansion out of a single refugium along the Atlantic Coast post-LGM is suggested by a phylogeographic study of *Ambystoma maculatum* (Zamudio and Savage, 2003). Applied to *N. viridescens*, this scenario would suggest that intraspecific ecological divergence occurred entirely during postglacial expansion and little effect of Pleistocene refugial use on subspecific divergence ($H_{1\text{-I}}$ in Fig. 2). Given the locations of the proposed Pleistocene refugia and the distribution pattern of the subspecies, any scenarios in which four subspecies independently arise from four different refugia are unrealistic. Since the Atlantic Coast refugium is most likely used by newts as in other amphibians widely distributed in eastern United States (Church et al., 2003; Zamudio and Savage, 2003), we generated two-refugium and three-refugium hypotheses by combining the Atlantic Coast refugium and one or two other potential refugia. As detailed in Fig. 2, support for any of six hypotheses ($H_{2\text{-IV}}$, $H_{3\text{-I}}$, $H_{3\text{-VI}}$, $H_{3\text{-VII}}$, $H_{4\text{-I}}$, $H_{4\text{-II}}$) would suggest that Pleistocene refugia and postglacial expansion together affected the formation of the newt subspecies. Support for any of the remaining hypotheses would suggest phylogenetic discordance with the classification of the eastern newt subspecies

Relaxed-clock Bayesian analyses were conducted in Beast (vers. 1.4.8; Drummond and Rambaut, 2007) to test the 16 phylogeographic predictions. The nucleotide dataset was partitioned into three subsets: for coding loci, a partition comprised first and second codon positions; a second partition comprised all third-positions in codons; the last partition included all non-coding sites. The general time-reversible model with gamma-distributed among-site rate-heterogeneity ($GTR + \Gamma$) with estimated base-frequencies was used to model sequence evolution for each partition. Model parameters were assumed to be independent among partitions. The Γ-distribution was discretized into four categories. Using an uncorrelated lognormal prior on among-branch rate-variation, fit of various coalescent models (i.e., constant size, logistic growth, expansion growth, exponential growth; see Drummond and Rambaut, 2007) were compared using $\log_{10}$ Bayes-factors (hereafter, BF_{log}; Jeffreys, 1961; Suchard et al., 2001) in a preliminary analysis. We follow Kass and Raftery (1995) in criteria used to judge relative support for hypotheses by Bayes factors, where a difference of $BF_{log} > 3$ is substantial evidence for the better supported hypothesis (i.e., with 1000-fold more support). Having chosen exponential growth as the most appropriate coalescent model by Bayes-factor comparisons, phylogeographic hypothesis-testing was conducted (see Fig. 2 for details of each hypothetical topologies). For topological tests hypothesizing coalescence for a set of haplotypes at the LGM, we constrain tree-searches such that node height of the root conformed to the prior distribution $\sim N(\mu = 35.2$ Mya, $\sigma^2 = 7.27)$, and node heights for *Notophthalmus* sampled from the prior distribution $\sim N(\mu = 11.4$ Mya, $\sigma^2 = 4.28)$. These distributions span posterior ranges reported by Zhang



Refugial Hypotheses	Single refugium (H_1)	Two refugia (H_2)					
	I	I	II	III	IV	V	VI
Potential refugia	(i)AC 🟫	(i) AC 🟫 (ii) eGC 🟩		(i) AC 🟫 (ii) App 🟦		(i) AC 🟫 (ii) wGC ⬛	
Patterns of post-glacial migration	(i) to entire current distribution range	(i) to entire AC, and App (*P, L, D, V*) (ii) to entire GC and IP (*L*)	(i) to entire AC (*P, L, D*) (ii) to entire GC, IP, and App (*L, V*)	(i) to entire AC (*P, L, D*) (ii) to entire App, GC and IP (*V, L*)	(i) to entire AC, GC, and IP (*P, L, D*) (ii) to entire App (*V*)	(i) to entire current distribution range except wGC (*P, L, D, V*) (ii) to entire wGC (*L*)	(i) to entire AC, and App (*P, L, D, V*) (ii) to entire GC and IP (*L*)
Glaciations affected ecological divergence among subspecies?	No	No	No	No	Partially possible (*V*)	No	No
Subspecies formed via post-glacial adaptive radiation?	Yes all	Yes all	Yes all	Yes all	Partially (*P, L, D*)	Yes all	Yes all
Subspecies monophyly posible?	Yes all	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes all	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)
Ecological niche analyses needed? (see Fig. 1)	No	No	No	No	Yes	No	No

Refugial Hypotheses	Three refugia (H_3)							Four refugia (H_4)	
	I	II	III	IV	V	VI	VII	I	II
Potential refugia	(i) AC 🟫 (ii) eGC 🟩 (iii) App 🟦	(i) AC 🟫 (ii) eGC 🟩 (iii) wGC ⬛				(i) AC 🟫 (ii) App 🟦 (iii) wGC ⬛		(i) AC 🟫 (ii) eGC 🟩 (iii)App 🟦 (iv) wGC ⬛	
Patterns of post-glacial migration	(i) to entir AC (*P, L, D*) (ii) to entir GC and IP (*L*) (iii) to entir App (*V*)	(i) to entir AC and App (*P, L, D, V*) (ii) to entir eGC and IP (*L*) (iii) to entir wGC (*L*)	(i) to entir AC and App (*P, L, D, V*) (ii) to entir eGC (*L*) (iii) to entir wGC and IP (*L*)	(i) to entir AC (*P, L, D*) (ii) to entir eGC, IP, and App (*L, V*) (iii) to entir wGC (*L*)	(i) to entir AC (*P, L, D*) (ii) to entir eGC and App (*L, V*) (iii) to entir wGC and IP (*L*)	(i) to entir AC (*P, L, D*) (ii) to entir App and eGC (*L, V*) (iii) to entir wGC and IP (*L*)	(i) to entir AC and eGC (*P, L, D*) (ii) to entir App (*V*) (iii) to entir wGC and IP (*L*)	(i) to entir AC (*P, L, D*) (ii) to entir eGC and IP (*L*) (iii) to entir App (*V*) (iv) to entir wGC (*L*)	(i) to entir AC (*P, L, D*) (ii) to entir eGC (*L*) (iii) to entir App (*V*) (iv) to entir wGC and IP (*L*)
Glaciations affected ecological divergence among subspecies?	Partially possible (*V*)	No	No	No	No	Partially possible (*V*)	Partially possible (*V*)	Partially possible (*V*)	Partially possible (*V*)
Subspecies formed via post-glacial adaptive radiation?	Partially (*P, L, D*)	Yes all	Yes all	esYall	Yes all	Partially (*P, L, D*)	Partially (*P, L, D*)	Partially (*P, L, D*)	Partially (*P, L, D*)
Subspecies monophyly posible?	Yes (*P, D, V*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)	Yes (*P, D, L*) No (*L* paraphyletic)
Ecological niche analyses needed? (see Fig. 1)	Yes	No	No	No	No	Yes	Yes	Yes	Yes

Fig. 2. A list of phylogeographically realistic predictions of *N. viridescens* topology that differ in the number and coarsely-defined location of Pleistocene refugia and the pattern of the postglacial expansion. These predictions were generated based on current knowledge on Pleistocene refugia and the distribution patterns of *N. viridescens* subspecies. Geographic regions are abbreviated as follows: AC = Atlantic Coastal Plain, APP = Appalachian Highlands, eGC = eastern Gulf Coastal Plains, IP = Interior Plains and wGC = western Gulf Coastal Plains (see Fig. 1).

et al. (2008) and incorporate an additional proportion (10 percent of the mean) added to each end of that range.

If reduced to relatively small population size, as might be expected for refugial populations, gene coalescence ought to occur rapidly (Kingman, 1982; Knowles and Maddison, 2002). We employ a *distribution* on the expected period of coalescence rather than constrain to a point estimate for the LGM to account for stochasticity of the coalescent process (Knowles et al., 2007). Thus, in testing for coalescences attributable to the LGM, node-height sampling for our constrained topological hypotheses conformed to the uniform distribution of the interval 0–18 kya.

To assess support for each hypothesis, four independent Markov-chain Monte Carlo (MCMC) analyses were run, the first three of which comprising 10^6 generations with subsampling every 10^3 generations; the fourth chain was 5×10^6 generations in length and was subsampled every 10^4 generations. Redundant haplotypes were retained to permit better estimates of coalescent parameters. Tracer (vers. 1.4.8; Drummond and Rambaut, 2007) was used to assess chain convergence, stationarity, and independence of MCMC sampling (evaluated by effective sample sizes, ESSs). If independent chains were not substantially different in marginal tree-likelihoods ($|BF_{\log}| \leqslant 0.5$), posterior samples from the four chains were pooled with LogCombiner (Drummond and Rambaut, 2007). To eliminate subjectivity, the first 1/5 of samples from each chain was discarded as burnin. Convergence in likelihoods appeared sufficient among chains, and stationarity appeared reached well before the end of the burnin period. Concatenated samples were then compared amongst all phylogeographic predictions, which included the posterior sample from a topologically-unconstrained analysis. Clade credibilities and summary trees were computed using TreeAnnotator (version 1.4.6; Drummond and Rambaut, 2007) from the posterior distribution of trees for the topologically-unconstrained analysis (hereafter UC_{pdt}) and for the most-preferred constrained search (hereafter, PC_{pdt}).

To attempt to replicate findings of subspecific non-monophyly (see Gabor and Nice, 2004), the UC_{pdt} was subjected to topological filters in Paup* (Swofford, 2000), with each filter-constraint consistent with the monophyly of a particular subspecies. Proportion of trees consistent with each filter-constraint was interpreted as the posterior probability (p_{pos}) of subspecific monophyly.

2.4. Tests of Pleistocene refugia and post-glacial expansion

The posterior distributions of trees retained from the preferred constrained-analysis in Beast were used to estimate likely geographic locations of coalescent events. If ancestors of particular clades shared Pleistocene refugia, gene coalescence ought to have occurred at around this period of severe range contraction (and presumed reduction in effective population size). Trees were pruned to leave only non-redundant haplotypes.

We used Phylomapper (vers. 1b1; Lemmon and Lemmon, 2008) to evaluate likely geographic positions of gene-coalescence events. Phylomapper implements a stochastic model of populational expansion across a landscape where geographic coordinates of sampled populations are assumed to evolve through the tree by Brownian motion (Lemmon and Lemmon, 2008). Given that genetic data are often spatially autocorrelated, this implementation and assumption seemed appropriate.

Ancestral coordinates were estimated for three non-nested clades receiving high posterior support in the PC_{pdt}. To accommodate topological uncertainty, locality estimates were computed across the posterior distribution of 10^4 samples, with tree samples drawn at random. In several cases, redundant haplotypes were sampled in geographically distinct areas. As Phylomapper does not permit zero-length edges (Lemmon and Lemmon, 2008), several locality datasets were necessary to accommodate 'uncertainty' in the geographical location of each haplotype if redundant. If redundant haplotypes were sampled within one degree of latitude and longitude, values amongst redundant haplotypes were averaged. Each potential combination of substantially different sampling-localities of redundant haplotypes was used, however, resulting in eight distinct locality datasets. For each dataset, 125 draws from the posterior distribution of trees were used for estimates of ancestral localities. To express variance in locality-estimates of coalescent events with respect to tree space, the R-package (R Development Core, 2008) car (Fox, 2002) was used to compute confidence ellipses for all phylogeographic groups of interest. All R scripts used herein are available upon request.

As spatial distribution of genetic data can inform historical patterns of movement and gene flow (Templeton et al., 1987; Knowles and Maddison, 2002), the methodological procedure outlined by Hafner et al. (2008) was adopted to assess radial orientation between redundant haplotypes. For instance, if all redundant haplotypes are sampled along a NW–SE axis, a principal route of historical migration is likely to have occurred along the same axis (Hafner et al., 2008). If a particular refugium was used by ancestors of a set of descendants, angular relationships between identical haplotypes should conform to the directionality of expansion from the refugial population – if migratory patterns are largely stable through time. Using the R-package CircStats (Jammalamadaka and SenGupta, 2001; Agostinelli, 2009), Rao's (1976) spacing test of uniformity was employed to determine if observed axial directions between redundant haplotypes provided signal for directionality of population expansion, evidenced where more clustering of axial connections occurs between haplotypes than is expected by chance. Detection of directional postglacial expansion would suggest possible biases in estimation of the LGM refugial locations shifted toward such direction.

2.5. Ecological niche analyses

To compare environmental conditions of fundamental niches between the modern and the LGM newt lineages, we first created ecological niche models (EMNs) for the modern subspecies, the modern phylogenetic lineages, and the LGM refugial populations. Because the subspecies designations were not congruent with the most preferred constrained tree from our phylogeographic testing, we conducted separate analyses on the subspecies and the modern lineages. The ENMs of the LGM populations were created based on ancestral coordinates estimated through Phylomapper and reconstructions of the LGM climatic variables. We then generated random points over each ecological niche model and extracted environmental variables by those random points. This process was critical in that it transformed environmental conditions represented only by occurrence and estimated ancestral coordinates into the broader-spectrum dataset that represents newt's fundamental niche conditions. Finally, to test whether the ecological niches of *N. viridescens* have shifted since the LGM, we performed principal component analyses on the extracted environmental variables and tested for differences using multivariate analyses of variance and environmental space overlaps.

We constructed EMNs of the modern and the LGM lineages of *N. viridescens* using Maxent version 3.3.3e (Phillips et al., 2006). We used Maxent's default settings for all parameters but chose cumulative output, which is interpreted as predicted suitable conditions for the organism above a threshold based on predicted omission rate. We chose cumulative output because our interest was in habitat suitability rather than probability of presence inferred from the default logistic output. For the modern subspecies locality data, we combined the locality records of our samples used in the phylogenetic analyses and museum records obtained through the HerpNet database (http://herpnet.org). For records lacking GPS coordinates, we georeferenced locations based on locality descriptions. We inferred the distribution ranges of the modern phylogenetic lineages for the assignment of the museum specimen locations by using ArcGIS (ESRI, 2012) to subdivide the *N. viridescens* species range based on spatial haplotype distributions derived from our phylogenetic analysis (Fig. 3). To create the lineage ranges, we gave each phylogenetic sample location a value of 1 if the lineage was present and 0 otherwise and used the Inverse Distance Weighted interpolation procedure in ArcGIS to calculate an inverse square surface for each lineage. Using a threshold value of 0.4, we converted the gridded interpolation surfaces to polygons, clipped each to the *N. viridescens* species range, and then extracted all museum specimen locations falling within each lineage polygon to a separate lineage point file. To construct the LGM refugial ENMs, we used ancestral coordinates estimated from the preferred constrained-analysis in Beast described above. To reduce the effects of spatial autocorrelation, we treated localities within 0.1° of one another as duplicate records, either of which was removed at random (Waltari et al., 2007). This results in occurrence data files with 660 modern subspecies occurrence records (20 *N. v. dorsalis*, 115 *N. v. louisianensis*, 21 *N. v. piaropicola*, and 504 *N. v. viridescens*), 677 modern lineage occurrence records (133 Eastern, 513 Appalachian Highland, and 31 Western Gulf Coast), and 716 LGM refugial locality points (214 Eastern, 362 Appalachian Highland, and 140 Western Gulf Coast). The total number of the modern lineage data points was greater than that of the subspecies because some locality points were used multiple times due to the lineage range overlaps (Fig. 3).

For environmental variables, we obtained gridded 2.5′ data sets representing modern and LGM (21 kya) climate conditions from the WorldClim database (Hijmans et al., 2005), which includes 19 bioclimatic variables (Table 2). The WorldClim LGM reconstructions were produced using the Community Climate System Model (CCSM, Collins et al., 2006) and the Model for Interdisciplinary Research on Climate (MIROC, Hasumi and Emori, 2004) for the Paleoclimate Modeliing Intercomparison Project Phase II (PMIP2 http://pmip2.lsce.ipsl.fr/). Because WorldClim bioclimate variables are often intercorrelated (e.g., Rissler and Apodaca, 2007), we generated correlation matrices to identify highly correlated (Pearson correlation coefficient >0.8) pairs and chose the most biologically meaningful and easily interpreted variables as Maxent inputs (Table 2). To determine whether the ENMs generated by Maxent were better than random predictions, the area under the Receiver Operating Characteristic curve (AUC) is calculated for each ENM. The AUC is a measure of model performance and varies from 0 to 1 with a value greater than 0.5 indicating that the model performance was better than random prediction and 1.0 for perfect predictability (Elith et al., 2006).

To prepare data sets for the principal components analysis, we converted the 13 gridded ENMs (4 subspecies, 3 modern lineages, 3 CCSM refugia, and 3 MIROC refugia) to polygons. To minimize

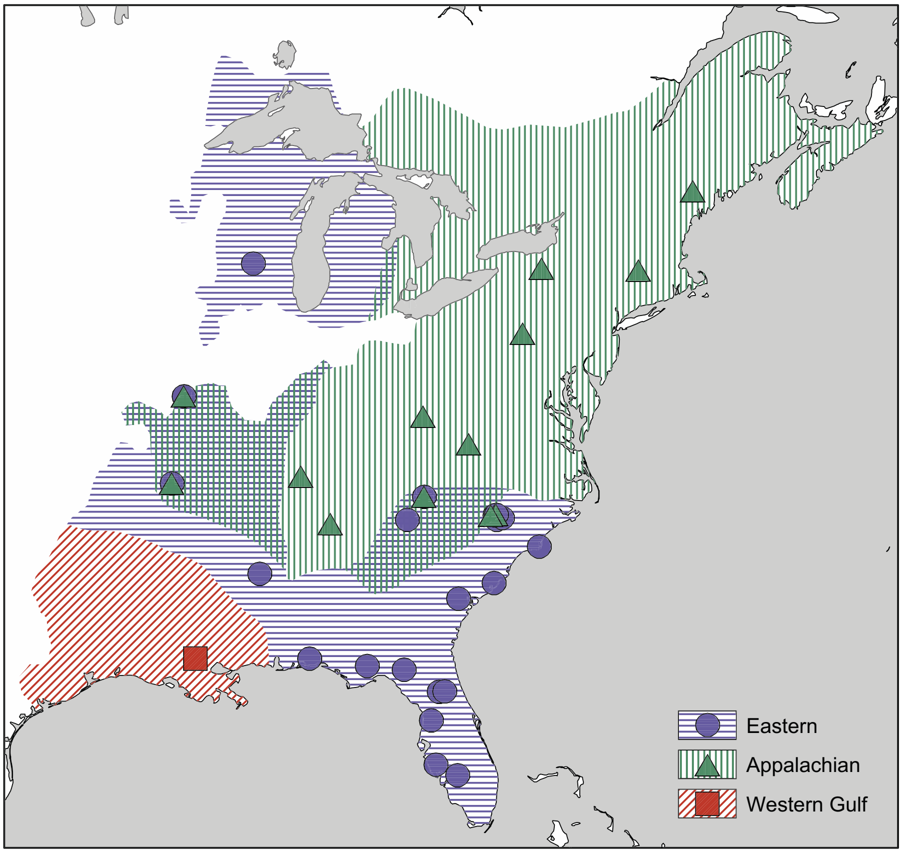


Fig. 3. Estimated distribution range of the modern phylogenetic lineages (Eastern, Appalachian Highland, and western Gulf Coast) of *N. viridescens* based on spatial haplotype distributions.

Table 2
List of 19 BIOCLIM variables and selected variables based on correlation matrices for the ecological niche modeling of the modern and LGM lineages. The LGM climatic variables are based on two reconstruction models, CCSM and MIROC (see Methods). These bioclimatic layers can be downloaded from Worldclim database (http://www.worldclim.org/bioclim).

Modern	LGM			
	CCSM	MIROC		
v	v	v	BIO 1	Annual mean temperature
v	v		BIO 2	Mean diurnal range (mean of monthly (max temp–min temp))
	v	v	BIO 3	Isothermality (BIO2/BIO7) (*100)
			BIO 4	Temperature seasonality (standard deviation *100)
			BIO 5	Max temperature of warmest month
			BIO 6	Min temperature of coldest month
			BIO 7	Temperature annual range (BIO5–BIO6)
v	v	v	BIO 8	Mean temperature of wettest quarter
	v	v	BIO 9	Mean temperature of driest quarter
			BIO 10	Mean temperature of warmest quarter
			BIO 11	Mean temperature of coldest quarter
v	v	v	BIO 12	Annual precipitation
			BIO 13	Precipitation of wettest month
v			BIO 14	Precipitation of driest month
v	v	v	BIO 15	Precipitation seasonality (coefficient of variation)
			BIO 16	Precipitation of wettest quarter
			BIO 17	Precipitation of driest quarter
v	v	v	BIO 18	Precipitation of warmest quarter
			BIO 19	Precipitation of coldest quarter

total errors resulting from both exclusion of potentially suitable areas and inclusion of potentially unsuitable areas, we used as a threshold the ENM value corresponding to the minimum value for the summed omission rate and the fractional value of predicted background provided in the Maxent output.

We then used a stratified systematic unaligned sampling process (Berry and Baker, 1968) to generate random locality points within 1000 km^2 grid cells within each of the 13 ENM range polygons and extracted the niche suitability score (i.e., cumulative output score) for each point. Subsequently, we conducted a weighted lottery to ensure that the density of random locality points was proportional to niche suitability (i.e., more points from more suitable habitats) by selecting locality points that had suitability scores greater than random suitability scores generated with the same mean and standard deviation as in the niche suitability scores. We then extracted bioclimatic values from all 19 layers for each selected locality point.

Using the extracted bioclimate values, we conducted principal component analyses (PCA) followed by multivariate analyses of variance (MANOVAs) to statistically evaluate the ecological niche divergence of the newt lineages since the LGM (e.g., Graham et al., 2004; Rissler and Apodaca, 2007). We conducted post hoc multiple pairwise comparisons using MANOVAs with Bonferroni corrections to test niche separation by the first two principal axes. With this analytical procedure, we first tested ecological niche divergence among the subspecies to confirm that 19 bioclimate variables demonstrated the predicted pattern of ecological divergence among the subspecies of *N. viridescens*. In a principal-component environmental space with the first two factors as axes, we predicted that (1) the most terrestrial subspecies *N. v. viridescens* and the most aquatic subspecies *N. v. piaropicola* would be most differentiated, (2) ecologically similar subspecies, *N. v. dorsalis* and *N. v. louisianensis*, would be nearest each other, and (3) these ecological similar subspecies that exhibit intermediate life history characteristics (see Introduction) would be placed between *N. v. viridescens* and *N. v. piaropicola*. Subsequently, we tested ecological niche divergence between the refugial populations and the subspecies using the modern and two LGM bioclimatic layers (CCSM and MICRO). We then repeated the same procedure to test niche divergence between the LGM refugial populations and the modern phylogenetic lineages. Finally, we calculated overlaps in principal-component (PC hereafter) environmental spaces between any possible pairs within each analysis as area of pair-wise overlap in 95% ellipses divided by the total area of the pair's 95% ellipses. We did not use statistical tools that test niche divergence based on ENM range overlaps (e.g., ENM Tools: Warren et al., 2008) because it is impossible to spatially compare between past and present distribution models.

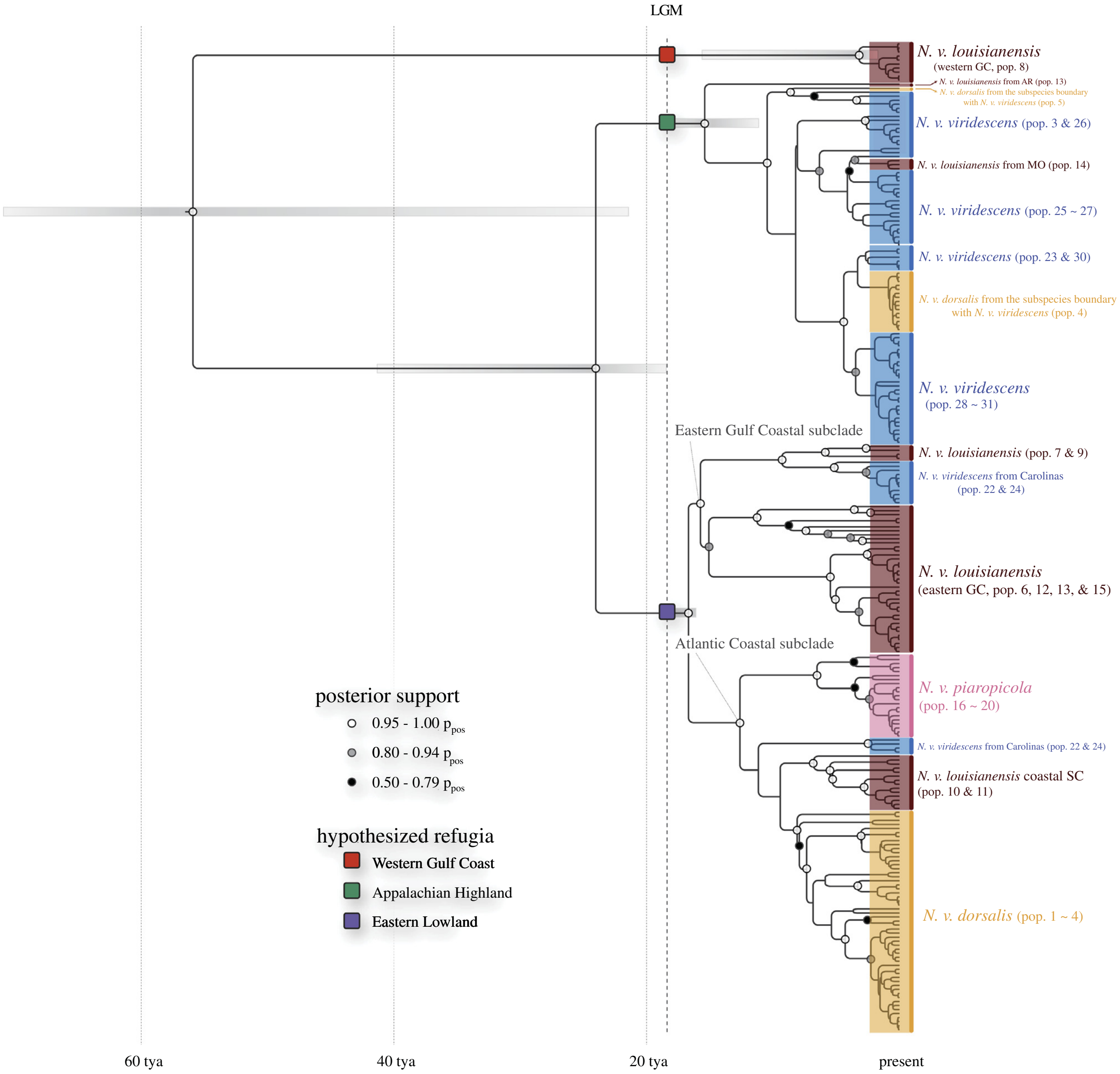


Fig. 4. The most preferred topological prediction of *N. viridescens* phylogeny ($H_{3\text{-VI}}$ in Fig. 2) by log Bayes-factors with nodal supports indicated by the Bayesian posterior probabilities. Three refugia were identified, namely Eastern Lowland, Appalachian Highland, and western Gulf Coast.

Table 3
Pair-wise comparisons of the refugial predictions based on log Bayes-factors (BF_{log}).

	BF_{log}	+/−	H_1I	H_2I	H_2II	H_2III	H_2IV	H_2V	H_2VI	H_3I	H_3II	H_3III	H_3IV	H_3V	H_3VI	H_3VII	H_4I	H_4II	Mean	Rank
H_1I	−6440.9	0.52	–	16.83	16.13	0.84	1.00	−2.20	1.07	16.28	6.88	16.00	10.00	16.67	−2.52	21.96	5.66	15.82	9.36	3
H_2I	−6479.7	0.31	−16.83	–	−0.71	−15.99	−15.83	−19.03	−15.76	−0.55	−9.95	−0.83	−6.83	-0.16	−19.35	5.13	−11.17	−1.01	−8.59	15
H_2II	−6478.0	0.33	−16.13	0.71	–	−15.29	−15.12	−18.32	−15.06	0.16	−9.25	−0.13	−6.13	0.54	−18.64	5.83	−10.46	−0.30	−7.84	12
H_2III	−6442.8	0.37	−0.84	15.99	15.29	–	0.16	−3.04	0.23	15.44	6.04	15.16	9.16	15.83	−3.36	21.12	4.83	14.98	8.46	4
H_2IV	−6443.2	0.30	−1.00	15.83	15.12	−0.16	–	−3.20	0.06	15.28	5.88	14.99	9.00	15.66	−3.52	20.96	4.66	14.82	8.29	5
H_2V	−6435.8	0.31	2.20	19.03	18.32	3.04	3.20	–	3.27	18.48	9.08	18.19	12.20	18.87	−0.32	24.16	7.86	18.02	11.71	2
H_2VI	−6443.4	0.32	−1.07	15.76	15.06	−0.23	−0.06	−3.27	–	15.21	5.81	14.93	8.93	15.60	−3.59	20.89	4.60	14.76	8.22	6
H_3I	−6478.4	0.39	−16.28	0.55	-0.16	−15.44	−15.28	−18.48	−15.21	–	−9.40	−0.29	−6.28	0.39	−18.80	5.68	−10.62	-0.46	−8.01	13
H_3II	−6456.7	0.32	−6.88	9.95	9.25	−6.04	−5.88	−9.08	−5.81	9.40	–	9.12	3.12	9.79	−9.40	15.08	−1.21	8.95	2.02	8
H_3III	−6477.7	0.32	−16.00	0.83	0.13	−15.16	−14.99	−18.19	−14.93	0.29	−9.12	-	−6.00	0.67	−18.52	5.96	−10.33	−0.17	−7.70	11
H_3IV	−6463.9	0.31	−10.00	6.83	6.13	−9.16	−9.00	−12.20	−8.93	6.28	−3.12	6.00	–	6.67	−12.52	11.96	−4.34	5.82	−1.31	9
H_3V	−6479.3	0.33	−16.67	0.16	−0.54	−15.83	−15.66	−18.87	−15.60	−0.39	−9.79	−0.67	−6.67	-	−19.19	5.29	−11.00	−0.84	−8.42	14
H_3VI	−6435.1	0.37	2.52	19.35	18.64	3.36	3.52	0.32	3.59	18.80	9.40	18.52	12.52	19.19	–	24.48	8.18	18.34	12.05	1
H_3VII	−6491.5	0.30	−21.96	−5.13	−5.83	−21.12	−20.96	−24.16	−20.89	−5.68	−15.08	−5.96	−11.96	−5.29	−24.48	–	−16.29	−6.14	−14.06	16
H_4I	−6454.0	0.33	−5.66	11.17	10.46	−4.83	−4.66	−7.86	−4.60	10.62	1.21	10.33	4.34	11.00	−8.18	16.29	–	10.16	3.32	7
H_4II	−6477.3	0.33	−15.82	1.01	0.30	−14.98	−14.82	−18.02	−14.76	0.46	−8.95	0.17	−5.82	0.84	−18.34	6.14	−10.16	–	−7.52	10

3. Results

3.1. Topological hypothesis-testing

We obtained maximal lengths of 1585 concatenated nucleotides of mtDNA sequence data for each of 246 individuals from 31 sampling localities. We identified 135 haplotypes (Cyt b GenBank ID: KJ776842 - KJ776972; ND2 GenBank ID: KJ776973 - KJ777103). Excluding the outgroup, there are 272 variable sites, among which 175 were parsimony-informative.

The topology consistent with $H_{3\text{-VI}}$ (Fig. 4) was most preferred by log Bayes-factors (Table 3) and was only slightly less supported than the topologically unconstrained analysis ($BF_{log} = 0.265$). The preferred constrained-analysis identified three major clades that were statistically well supported: Appalachian Highlands, Eastern Lowland, and Western Gulf Coast, corresponding to the three hypothesized refugia in these areas (Fig. 5). The Eastern Lowland clade is further split into two geographically cohesive units, the Eastern Gulf Coastal and the Atlantic Coastal subclades.

The Appalachian Highland refugial population has predominantly formed *N. v. viridescens* with a few exceptions. Population 22 of *N. v. viridescens* in the Blue Ridge Mountains exclusively comprises haplotypes belonging to the Eastern Gulf Coastal subclade, while the nearby population 24 of *N. v. viridescens* consists of both the Eastern Gulf Coastal subclade and the Appalachian Highland clade (Table 1, Fig. 4). The phylogenetic congruence between the Appalachian Highland refugial population and the majority of *N. v. viridescens* suggests that ecological divergence of this most terrestrial subspecies may have occurred via adaptation to the Appalachian Highland refugium during the LGM. This possibility was tested by the ecological niche analyses (see below).

The remaining inferred refugial lineages (Eastern Lowland and Western Gulf Coast) did not perfectly correspond with *N. v. dorsalis*, *louisianensis*, or *piaropicola*. The Atlantic Coastal subclade comprises haplotypes from all four subspecies, within which *N. v. dorsalis* and *N. v. piaropicola* each form well-supported monophyletic groups. The populations of *N. v. louisianensis* within the Atlantic Coastal subclade are all located east of the Suwannee River. The Eastern Gulf Coastal subclade consists predominantly of *N. v. louisianensis* distributed across the eastern Gulf coast and the Interior Plains but also *N. viridescens* from the mountains of North and South Carolina (Table 1). Finally, the Western Gulf Coastal clade exclusively consists of haplotypes of *N. v. louisianensis* from Louisiana.

Monophyly was strongly supported for *N. v. piaropicola* (Beast $p_{pos} = 1.000$) and fully lacking for *N. v. viridescens*, *N. v. dorsalis* and *N. v. louisianensis* (i.e., all Beast $p_{pos} = 0.000$). Two haplotypes sampled from populations of *N. v. dorsalis* occur along the subspecies boundary with *N. v. viridescens* appear to drive the non-monophyly of *N. v. dorsalis* (NC *viridescens* 8 and NC *dorsalis* 30 in Table 1). These haplotypes of *N. v. viridescens* found in the *N. v. dorsalis* side of the subspecific boundary have likely resulted from gene flow from *N. v. viridescens*. If these two haplotypes are allowed to be positionally unconstrained, support for monophyly in *N. v. dorsalis* is strong (Beast $p_{pos} = 1.000$).

3.2. Pleistocene refugia and post-glacial expansion

The geography of estimated coalescent events at the root of each phylogeographic group is largely congruent with results from topological tests of phylogeography (Fig. 5). Coalescent areas are geographically distinct, suggesting possible genetic differentiation of *N. viridescens* through much of the Pleistocene glacial periods. These coalescent areas are interpreted as likely sites for refugia at the LGM, at least for the Appalachian Highland and Eastern Lowland lineages, whose coalescent timing overlaps the LGM (Fig. 4).

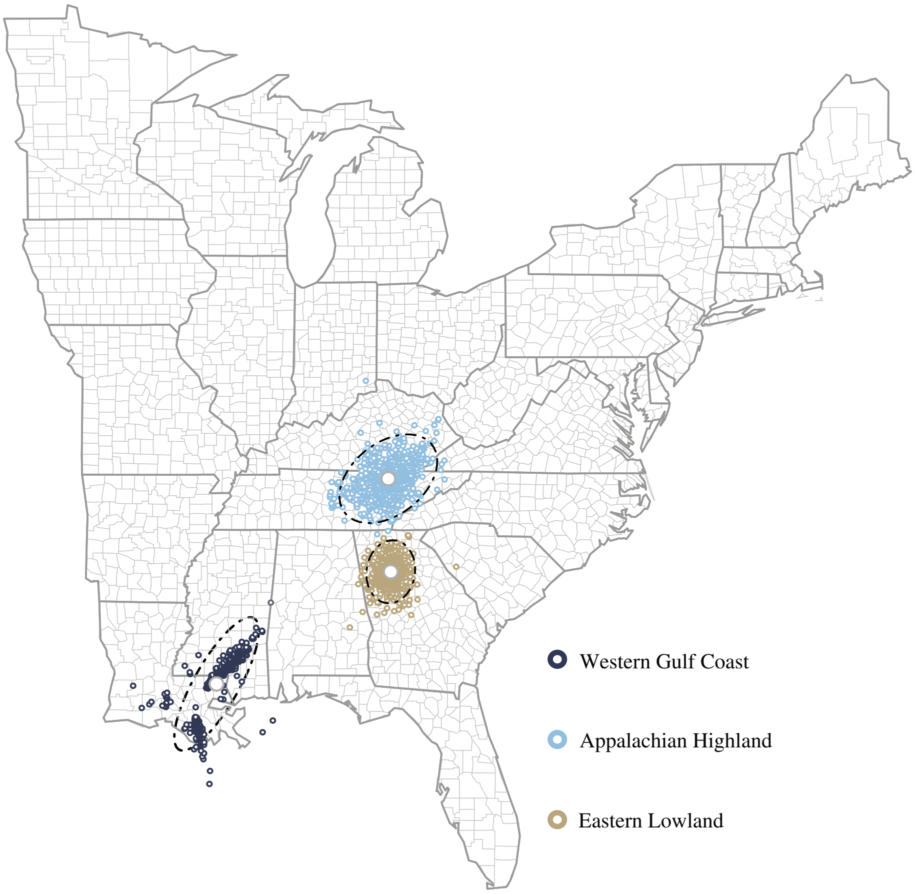


Fig. 5. Estimated ancestral geographic coordinates with confidence ellipses representing three potential LGM refugia for *N. viridescens*, Atlantic, Appalachian Highland, and western Gulf Coast.

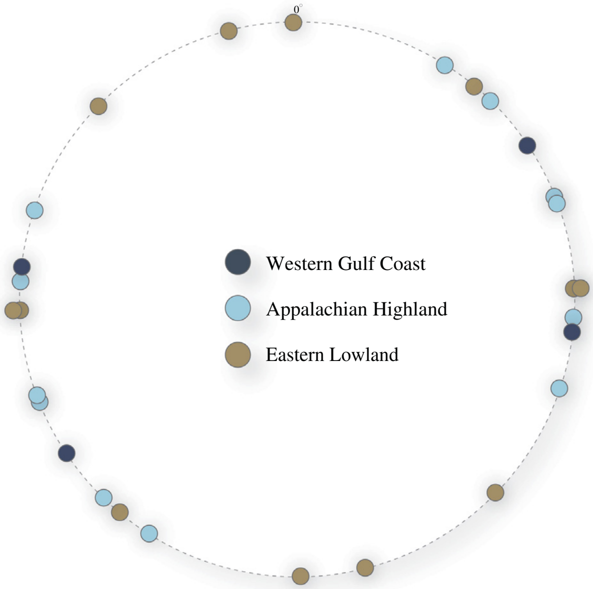


Fig. 6. The directional patterns of postglacial migration from each of the three LGM refugia that were detected in the preferred phylogenetic tree of *N. viridescens* (see Fig. 4). These migration patterns were estimated based on radial orientation between redundant haplotypes. For instance, if all redundant haplotypes are sampled along a N–S axis, a principal route of postglacial migration is likely to have occurred along the same axis. In our analyses, we found no cases were significant departures from expectations of randomness, suggesting that there were no directional postglacial migrations.

Using Hafner et al.'s method (2008) for assessing radial orientation between redundant haplotypes, there were no significant departures from expectations of randomness detected in tests of radial directionality for these three phylogeographic groups (Rao's U; $p > 0.10$; Fig. 6).

3.3. Ecological niche analyses

The AUCs of all ENMs were close to 1.0 (i.e., perfect predictability) with 0.973 being the lowest AUC, suggesting that our ENMs were robust. PCA using 19 BIOCLIM variables supported the predicted pattern of ecological divergence among the subspecies of *N. viridescens* in PC space with the first two factors (Wilks' lambda: $F = 88.15$, $P < 0.0001$, Fig. 7a). The most terrestrial subspecies *N. v. viridescens* and the most aquatic subspecies *N. v. piaropicola* are placed farthest from each other in environmental space with *N. v. louisianensis* and with *N. v. dorsalis* falling between them. The latter two subspecies share similar life history characteristics that are intermediate between *N. v. viridescens* and *N. v. piaropicola* (see Introduction). The results of the pairwise comparisons suggest that, overall, each subspecies occupies distinct niche while *N. v. dorsalis* and *N. v. louisianensis* did not differ along the second principal component axis (Fig. 7a). Loadings are summarized along the axes in Fig. 7a. On average, there is 8.9% overlap in 95% ellipses of PC environmental spaces between the subspecies (Table 4a).

PCA–MANOVA analyses based on both CCSM and MIROC paleoclimatic reconstructions revealed significant niche divergence between the subspecies and the refugial populations (CCSM: Wilks' lambda: $F = 224.99$, $P < 0.0001$, Fig. 7b; MIROC: Wilks' lambda:

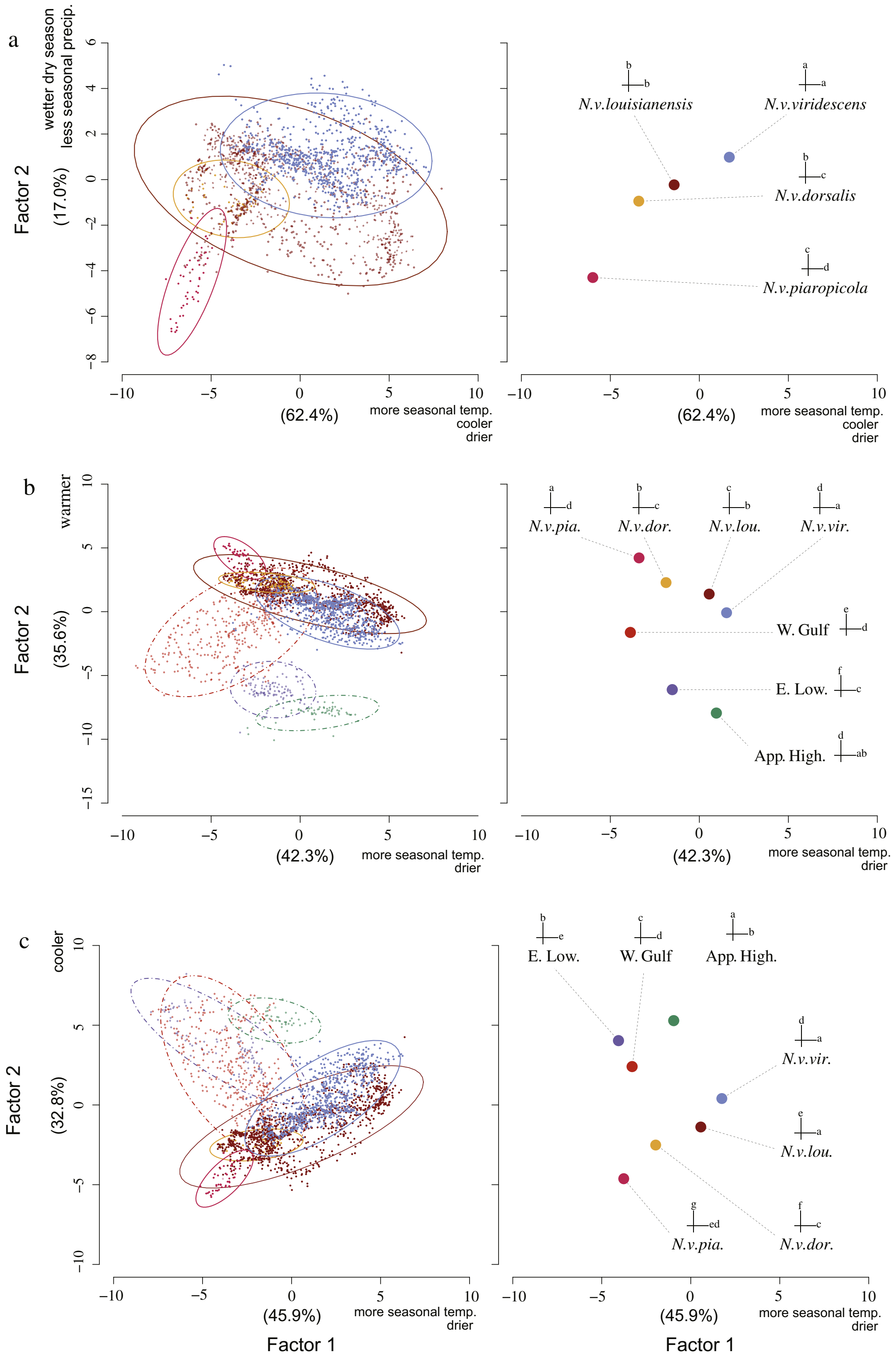


Fig. 7. Ecological niche divergence among (a) modern subspecies of *N. viridescens*, (b) modern subspecies (solid circles and ellipses) and refugial populations (open circles and dashed ellipses) based on CCSM paleoclimatic reconstruction, and (c) modern subspecies (solid circles and ellipses) and refugial populations (open circles and dashed ellipses) based on MIROC paleoclimatic reconstruction. The left-side panels show raw data with 95% ellipses. The right-side panels show group means with the results of the post hoc tests in which letters indicate statistical significance along each axis. For example, in the panel (a) there is no difference between *N. v. louisianensis* and *N. v. dorsalis* along the second-factor axis as letters along the *Y* axis in the cross next to those subspecies indicate "b" for both *N. v. louisianensis* and *N. v. dorsalis*; meanwhile, these two species differ along the first-factor axis ("b" for *N. v. louisianensis* and "c" for *N. v. dorsalis*). Factor loadings are interpreted and summarized along the axes of the left-side panels. Percentages of variance explained by the first and second factors are placed in parentheses along corresponding axes.

$F = 132.14$, $P < 0.0001$, Fig. 7c). On average, the 95% confidence ellipses for subspecies overlap those of the refugial populations only by 2.9% for the CCSM reconstruction and 3.3% for MIROC (Table 4b and c). In contrast, average overlaps within subspecies (10.5% in CCSM, 11.1% in MIROC) and within refugial populations (3.8% in CCSM, 14.1% in MIROC) are relatively high. In both CCSM and MIROC analyses, the PC 1 axis is primarily explained by seasonality in temperature and precipitation. The PC 2 is explained

Table 4
Pairwise overlaps of 95% ellipses in principal-component environmental spaces. Sub-tables a, b, and c correspond to Fig. 7a–c while sub-tables d–f correspond to Fig. 8a–c respectively.

a. Subspecies

	Nvd	*Nvl*	*Nvv*	*Nvp*
Nvd		25.9	14.4	1.9
Nvl			6.3	0.0
Nvv				4.7
Nvp				

Ave. overlap = 8.9%

b. Subspecies vs. CCSM Refugia

	Nvd	*Nvl*	*Nvv*	*Nvp*	CCSM-E. Low	CCSM-App. High	CCSM-W. Gulf
Nvd		29.0	12.1	6.9	0.0	0.0	14.0
Nvl			9.6	0.0	0.0	0.0	11.7
Nvv				5.3	0.0	0.0	8.5
Nvp					0.0	0.0	1.1
CCSM-E. Low						9.1	2.4
CCSM-App. High							0.0
CCSM-W. Gulf							

Ave. overlap between modern and LGM = 2.9%

c. Subspecies vs. MIROC Refugia

	Nvd	*Nvl*	*Nvv*	*Nvp*	MIROC-E. Low	MIROC-App. High	MIROC-W. Gulf
Nvd		29.2	12.4	7.2	1.8	0.0	10.6
Nvl			10.0	1.9	4.2	0.0	12.4
Nvv				5.9	3.0	0.0	7.8
Nvp					0.0	0.0	0.0
MIROC-E. Low						3.9	32.8
MIROC-App. High							5.7
MIROC-W. Gulf							

Ave. overlap between modern and LGM = 3.3%

d. Lineages

	E. Low	App. High	W. Gulf
E. Low		26.3	16.1
App. High			16.2
W. Gulf			

Ave. overlap = 19.5 %

e. Modern Lineages vs. CCSM Refugia

	E. Low	App. High	W. Gulf	CCSM-E. Low	CCSM-App. High	CCSM-W. Gulf
E. Low		34.6	23.4	0.0	0.0	16.6
App. High			15.2	0.0	0.0	11.9
Western Gulf				0.0	0.0	16.7
MIROC-East					9.1	2.9
CCSM-App. High						0.0
CCSM-W. Gulf						

Ave. overlap between modern and LGM = 5.0%

f. Modern Lineages vs. MIROC Refugia

	E. Low	App. High	W. Gulf	MIROC-E. Low	MIROC-App. High	MIROC-W. Gulf
E. Low		35.2	21.6	4.0	0.0	14.3
App. High			14.4	5.6	0.0	12.8
Western Gulf				0.0	0.0	14.0
MIROC-East					3.8	35.6
MIROC-App. High						5.5
MIROC-W. Gulf						

Ave. overlap between modern and LGM = 5.6%

by temperature-related variables; climatic environments of the refugial populations were cooler than those of the subspecies. While the CCSM and MIROC analyses showed similar results, there are a few major differences. First, the environmental niches of the refugial populations overlap more in the MIROC analysis than in the CCSM analysis. Second, while the PC 2 axis is positively associated with temperature-related variables in the CCSM analysis, the PC 2 axis in the MIROC analysis is negatively associated with temperature-relative variables.

MANOVAs revealed statistical differences among the modern phylogenetic lineages (Wilks' lambda: $F = 73.68$, $P < 0.0001$, Fig. 8a); however, their ecological niches are much less differentiated than those of the subspecies (8.9% overlap between the subspecies vs. 19.5% overlap between the lineages, Table 4a and d, respectively). Both CCSM and MIROC analyses suggest significant niche shift between the historical and modern lineages with the overall shift in temperature-related PC 2 axis to warmer conditions (CCSM: Wilks' lambda: $F = 293.47$, $P < 0.0001$, Fig. 8b; MIROC: Wilks' lambda: $F = 151.38$, $P < 0.0001$, Fig. 8c). Once again, the overlap between the historical and modern lineages in PC environmental spaces is relatively small (5.0% in CCSM and 5.6% MIROC, Table 4e and f), while the average overlaps within modern lineages (24.4% in CCSM and 23.7% in MIROC) and within refugial populations (4.0% in CCSM and 15.0% in MIROC) are relatively high. The notable difference between the CCSM and MIROC analyses is that the PC 2 axis in the CCSM analysis is positively associated with temperature-related variables while that in the MIROC analysis is negatively associated with temperature-related variables.

4. Discussion

By using *N. viridescens* as a model, the present study shows that the analytical framework that integrates phylogeographic tools and ecological niche analyses of current and past lineages without assuming niche stability is effective in exploring the historic processes driving the current pattern of ecological divergence. When tested as a hypothesis, rather than used as an assumption, our work emphasizes that the niches of *N. viridescens* lineages have been unstable since the LGM. Although the LGM refugial retreat had a major impact on the spatial distribution of genetic variation within *N. viridescens*, our data suggest that this vicariance event had a little effect on the currently-observed pattern of ecological divergence within *N. viridescens*. That is, postglacial adaptive expansion to the current habitats has likely resulted in the ecological divergence among the newt subspecies, instead of ecological differentiation having persisted since the LGM refugial populations. Thus, the present study points the importance of environmental characteristics, rather than vicariance events, in facilitating ecological divergence within wide-ranging species.

4.1. Pleistocene Refugia, phylogeographic patterns, and subspecies formation

Our evaluation of the 16 phylogeographic predictions suggests that *N. viridescens* most likely retreated to at least three refugia during the LGM, a Western Gulf Coast (southern Mississippi and

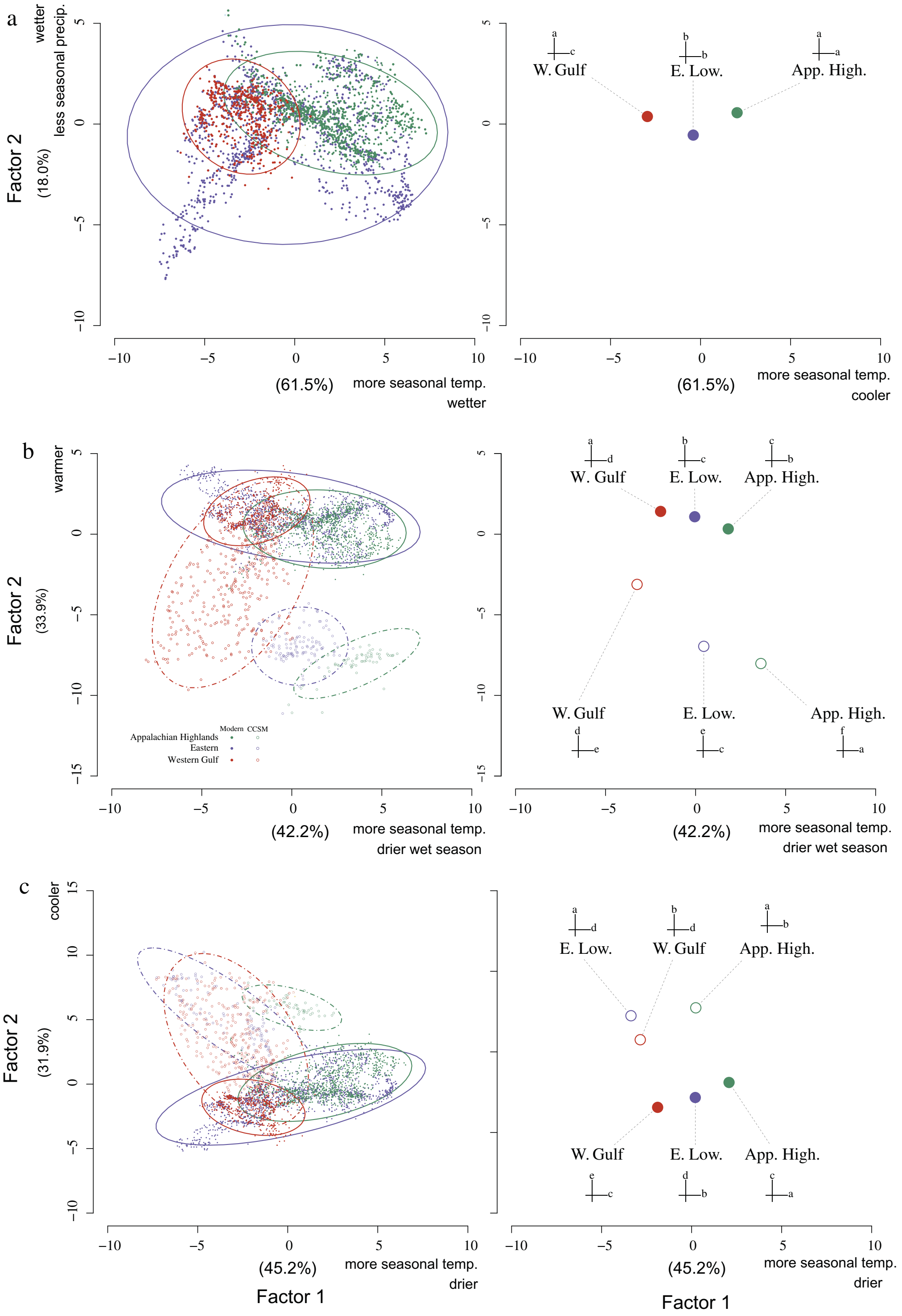


Fig. 8. Ecological niche divergence among (a) modern phylogenetic lineages of *N. viridescens*, (b) modern phylogenetic lineages (solid circles and ellipses) and refugial populations (open circles and dashed ellipses) based on CCSM paleoclimatic reconstruction, and (c) modern phylogenetic lineages (solid circles and ellipses) and refugial populations (open circles and dashed ellipses) based on MIROC paleoclimatic reconstruction. Graphical conventions follow those for Fig. 7.

Louisiana) refugium, an Appalachian Highland (southern Kentucky and eastern Tennessee) refugium, and an Eastern Lowland (northern Georgia) refugium (Fig. 5). The role of Pleistocene glaciations in forming intraspecific lineage sorting has been variable, although some congruent phylogenetic patterns still exist (Austin et al., 2004). The presence of southern Appalachian refugia (i.e., Appalachian Highland refugium in our study) is consistent with that reported for the eastern tiger salamander *Ambystoma t. tigrinum* (Church et al., 2003), spotted salamander *A. maculatum* (Zamudio and Savage, 2003), and spring peeper *Pseudacris crucifer* (Austin et al., 2004), all of which are widely distributed across the eastern United States and have geographic ranges that overlap extensively with that of *N. viridescens*. One study (Austin et al., 2004) that examined two anuran species also identified the Western Gulf Coast (i.e., Louisiana and its vicinity) and the area overlapping with our Eastern Lowland refugium (northern Georgia) as Pleistocene refugia for the bullfrog, *Lithobates catesbeiana*. These works suggest that wide-ranging amphibians in the eastern United States commonly retreated to southern Appalachian refugia, while retreats to refugia in Louisiana and northern Georgia appear to vary depending on species.

Three fragmented populations of *N. viridescens* between the Atlantic and the Gulf of Mexico had likely persisted in isolation during the LGM until postglacial warming allowed them to expand to their current distribution ranges. Secondary contact zones between *N. v. viridescens* and surrounding subspecies have likely formed via postglacial range expansion. In *N. v. viridescens*, populations in Pennsylvania, New York, Massachusetts, and Maine share identical haplotypes (Table 1), suggesting that northward migration from the Appalachian Highland refugium occurred rapidly as glaciers began retreating from these areas 18,000 kya (Pielou, 1991).

Modern river systems often remain barriers to contemporary gene flow of amphibian populations (Lemmon et al., 2007; Pauly et al., 2007), and the Apalachicola and Suwannee river systems appear to have been particularly important in fragmenting species into separate Atlantic and eastern Gulf Coast refugia during the LGM (Avise, 1992; Duellman, 1999; Church et al., 2003; Pauly et al., 2007). In *N. viridescens*, however, we estimated the genetic divergence between Atlantic – Gulf Coast lineages have occurred during postglacial expansion, rather than during the LGM (Fig. 4). The Suwannee River has likely been the major landscape barrier reducing gene flow between the Atlantic Coastal and Gulf Coastal lineages within one of the subspecies, *N. v. louisianensis*.

While the majority of current populations of *N. v. viridescens* have seemingly originated from the Appalachian Highland refugium, the multiple haplotypes found in populations 22 and 24 (Fig. 4, Table 1) represent two more distinct lineages contributing to the formation of this subspecies. One of these lineages originated from the Atlantic Coastal subclade, the other from the Gulf Coastal subclade, each of which formed a well-supported clade within the Eastern Lowland refugial lineage. One possibility of this finding, which needs further investigation with nuclear markers, is that these two lineages independently migrated into the Appalachian Highland during postglacial expansion and joined not only with each other but also with the Appalachian Highland lineage. Dorsal patterning of populations 22 and 24 is typical of *N. v. viridescens* (personal observation, MK Takahashi), and outdoor mesocosm experiments revealed that population 24 displayed the typical life history of *N. v. viridescens* with rapid and obligate metamorphosis to terrestrial efts (Takahashi and Parris, unpublished data). Therefore, it is possible that adaptation to the current niche of *N. v. viridescens* has occurred at least three times independently, suggesting the potential role of parallel evolution in different lineages.

Multiple distinct haplotypes found in two populations of *N. v. louisianensis* may also suggest such convergent evolution. While the Gulf Coastal subclade represents the vast majority of *N. v. louisianensis* west of the Suwannee River, populations 13 and 14 in Arkansas and Missouri contain both haplotypes of the Gulf Coastal subclade and Appalachian Highland clade (Fig. 4, Table 1), suggesting that a portion of the Appalachian Highland lineage has assumedly migrated and became part of *N. v. louisianensis* via adaptation to the Interior Plains and the Ozark Plateaus.

4.2. Effect of glaciations and the origin of ecological niche divergence

Overall, our phylogeographic and ENM analyses suggest the importance of ecology, rather than vicariance, in driving morphological and life history divergence within *N. viridescens*. Divergent natural selection can facilitate the evolution of reproductive isolation, leading to ecological speciation (Rundle and Nosil, 2005; Nosil et al., 2009; Schluter, 2009). Divergent selection favoring different life history strategies among the newt subspecies likely influences subspecific variation in body size, which may play a role in potential assortative mating. *Notophthalmus. v. viridescens* reach larger adult sizes by obligately metamorphosing to terrestrial efts and exploiting rich woodland environments for longer periods (i.e., typically 3–7 yr) than the other subspecies. In contrast, the other subspecies directly mature into smaller adults over short periods by remaining in aquatic habitats (5–7 mon.; Harris, 1987; Takahashi and Parris, 2008). Even when these subspecies metamorphose into terrestrial efts, their terrestrial stages are typically short ($\sim$1 yr; Harris et al., 1988), resulting in smaller adults. The short terrestrial stage may be an adaptation to the Coastal Plains' sandy soils and hot, dry terrestrial environment. As a result, body size difference is apparent between *N. v. viridescens* and the geographically proximal subspecies, *N. v. louisianensis* and *N. v. dorsalis*. Previous studies found that male newts prefer larger females because of fecundity advantages (Verrell, 1985, 1986), and larger males gain prior access to larger females by winning male-male competition (Verrell, 1986; Gabor et al., 2000), suggesting possibility of body-size assortative mating. However, a recent study showed that body-size assortative mating is probably not occurring (Takahashi et al., 2010). While larger males of *N. v. viridescens* gain prior access to larger females of their own kind, males of smaller subspecies still have chances to mate with female *N. v. viridescens* through sexual interference during spermatophore deposition of larger males. Thus, natural selection is likely to be a major force driving and maintaining ecological divergence among the newt subspecies while sexual selection may be halting complete ecological speciation.

Testing phylogenetic congruence between a vicariance event and coalescence timing has been a powerful method in inferring the influence of vicariance on speciation processes (Avise, 1992; Brant and Orti, 2003; Church et al., 2003; Lemmon et al., 2007; Spellman et al., 2007). In this study, we added biological and phylogeographic realism to such a phylogenetic analysis by generating and testing a nearly exhaustive list of topological scenarios based on the estimated period of the LGM, the current understanding of Pleistocene refugial locations, and contemporary *N. viridescens* distribution patterns. Our phylogenetic analysis supported the scenario in which the vast majority of *N. v. viridescens* is derived from one of three Pleistocene refugia (i.e., the Appalachian Highland refugium, Fig. 5), while the diversification of the other recognized subspecies could not be attributed to any single refugial population. These data suggest that ecological differentiation of *N. v. viridescens* from the rest of the subspecies (see Fig. 7a) may have occurred via adaptation to the Appalachian Highland refugial environment in allopatry during the LGM. However, our ecological niche analyses show significant differences in fundamental niches and no overlaps in 95% ellipses in PC environmental spaces between *N. v. viridescens* and the Appalachian Highland refugium (Fig. 7b and c; Table 4b and c). There is also little overlap in PC environmental space between the modern subspecies and the LGM populations, suggesting a niche shift since the LGM. Finally, the comparisons in ENMs show that ecological niches have become more overlapped among the modern than the historical lineages (Table 4, Fig. 8) and there is more overlap in PC environmental space among the modern lineages than among the subspecies (Table 4, Fig. 7a, Fig. 8a). These results suggest that: (1) Pleistocene glaciations had little effect on the current pattern of ecological niche divergence within *N. viridescens*; (2) the ecological divergence within the modern *N. viridescens* has occurred recently and has likely formed during postglacial range expansion; (3) the unit of divergence is likely to be the currently-recognized subspecies rather than the phylogenetic lineages; and 4) a marked niche shift of *N.* viridescens, particularly along temperature-related axis, has occurred since the LGM.

Poor estimation of Pleistocene refugial locations could also explain the niche divergence in PC environmental space between the current and the refugial populations of *N. viridescens*. However, we think that this interpretation is unlikely for three reasons. First, our estimated refugial locations are supported by previous amphibian studies (Appalachian Highland: tiger salamanders-Church et al., 2003; spotted salamanders-Zamudio and Savage, 2003; spring peeper-Austin et al., 2004; Atlantic Coast: bullfrog-Austin et al., 2004; Western Gulf Coast: bullfrog-Austin et al., 2004). Second, estimation of Pleistocene refugial locations via phylogeographic analyses is based on the assumption that the refugia existed within the current distributional range. This assumption is likely accurate given the broad geographic extent of *N. viridescens* likely encompassing the hypothesized Pleistocene refugia. And third, estimated patterns of postglacial migration from all three refugia were apparently random. Directional migration during postglacial expansion could have biased the estimation of Pleistocene refugial locations by shifting the centers of genetic diversity; however, such patterns of expansion were not detected in our analyses.

4.3. Current and LGM climatic conditions and life history evolution

The contemporary ecological niche of the most terrestrial subspecies (*N. v. viridescens*) is characterized in PC environmental space by cooler and drier environments with less seasonal precipitation as compared to that of the most aquatic subspecies (*N. v. piaropicola*) which are characterized by warmer and wetter environments with greater precipitation (Fig. 7a–c). The ecologically similar subspecies, *N. v. louisianensis* and *N. v. dorsalis*, utilize both terrestrial and aquatic habitats, and their niches fall in between the former two species. These niche characteristics at least partially explain the pattern of life history variation among the newt subspecies. As evidenced in the abundance and diversity of direct-developing, terrestrial plethodontid salamanders (Wilbur and Collins, 1973; Kozak and Wiens, 2010), the cool, high altitude, and persistently moist environment of the Appalachian Highlands may be one of the characteristics favoring a terrestrial life history among salamanders. On the other hand, greater precipitation likely contributes to permanence or longer persistence of freshwater habitats while severe dry seasons make terrestrial habitats unsuitable for salamanders. Thus, greater availability of permanent and long-standing aquatic habitat together with severe dry season likely selects for permanent (*N. v. piaropicola*) or plastic (*N. v. dorsalis* and *N. v. louisianensis*) aquatic life history in Florida and in the Coastal and Interior Plains.

Because there is little overlap in ecological niches between the subspecies and the refugial populations, it is difficult to predict life histories of the LGM refugial populations. Yet, the much colder environment of the refugial populations suggests that they may have been terrestrial because freshwater aquatic habitats, especially small and shallow fishless ponds, were likely to freeze in the winter months during the LGM. *Notophthalmus viridescens* is not freeze-tolerant and dies after 4 h of freezing at −2.5 °C (Storey and Storey, 1992). Thus, the effect of winter freeze on newt life history is functionally equivalent to pond desiccation. Winter-freeze avoidance behavior was observed in mountain Virginia, in which newts repeatedly migrated from permanent ponds to terrestrial winter hibernacula (Gill, 1978). While the modern ecological niche of *N. v. viridescens* is markedly different from that of the Appalachian Highland refugium especially along the temperature-related axis, adaptation to the refugial environment may have facilitated subsequent expansion of *N. v. viridescens* to the current habitats via a pre-adapted terrestrial life history.

As an environment undergoes significant changes, a population migrates, adapts, or goes extinct. Without an approach free from the niche-stability assumption, it is extremely difficult to test the possibility of adaptation. Our approach enables us to examine not only niche stability but also how the pattern of niche divergence has changed over time, providing a vital tool for understanding of the central question in evolutionary ecology.

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