

Conservation genetics of an endemic mountaintop salamander with an extremely limited range

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Abstract Montane regions can promote allopatric speciation and harbor unique species with small ranges. The southern Appalachians are a biodiversity hotspot for salamanders, and several montane endemics occur in the region. Here, we present the first DNA sequence data for *Plethodon sherando*, a terrestrial salamander recently discovered in the Blue Ridge Mountains of Virginia. We sequenced two mitochondrial regions (cyt-b and CO1) from salamanders at reference sites near the center of *P. sherando*'s range and from two contact zones where *P. sherando* populations are replaced by *Plethodon cinereus*, the Northern Red-Backed salamander. We then used these sequence data to examine divergence and hybridization between the two taxa. We found *P. sherando* and *P. cinereus* morphotypes from contact zones to be reciprocally monophyletic and highly divergent (~17%). *P. sherando* exhibited very low sequence diversity ($\pi = 0.0010$) as compared to *P. cinereus* from the same locations ($\pi = 0.0096$). Salamander morphology in the contact zone was as distinct as morphology at reference sites, and discriminant function analysis based on morphology successfully classified 98% of salamanders to their mitochondrial lineage. Phylogenetic analysis of cyt-b sequences showed *P. sherando* to be sister to *Plethodon serratus* (the Southern Red-Backed salamander) rather than *P. cinereus* or any nearby mountaintop endemics. Our results suggest that *P. sherando* is a distinct lineage that is not subject to substantial introgression from *P. cinereus* and that may have a history of geographic isolation. Given

its limited range (<80 km²), we believe *P. sherando* should merit a conservation status similar to that of other mountaintop salamanders in the region.

Keywords *Plethodon sherando* · Big Levels salamander · *Plethodon cinereus* · mtDNA · Phylogeography · Population genetics

Introduction

Montane regions are important biodiversity hot spots for a variety of plant and animal groups (Anderson and Maldonado-Ocampo 2010; McCain 2005; Myers et al. 2000). Mountain ecosystems can also be important factors in the creation of biodiversity, with mountain ranges providing opportunities for allopatric speciation (Knowles 2001; Steele et al. 2005; Xu et al. 2010). This may be particularly true in the tropics, where changes in elevation have been shown to correlate with different species assemblages or phenotypic traits (e.g. Lovett 1998; Terborgh 1977; Kleindorfer et al. 2006). However, temperate montane regions can also provide opportunities for speciation, and many species are endemic to montane regions of the temperate zone (Kozak and Wiens 2006).

Montane endemics may present particular challenges for conservation. For one, these species may be very susceptible to climate change since they cannot easily shift their ranges northwards or upslope in response to climate warming (Lawler et al. 2009; Moritz et al. 2008; Wilson et al. 2007). Studies that model shifts in habitat distribution under climate change projections generally project range contractions or extinctions for montane species (LaSorte and Jetz 2010; Milanovich et al. 2010). And for many montane species, replacement by competing species with

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higher thermal tolerances is potentially an even greater threat than simple change in the distribution of suitable habitats (Gilman et al. 2010).

In addition, mountaintop endemics, like other spatially-restricted species, may be susceptible to genetic swamping if they hybridize more widespread species. Gene flow at the boundaries of two related species is relatively common and does not necessarily threaten the coexistence of the two species (Colliard et al. 2010; Mallet 2005; Mallet et al. 2007). However, in the case of rare or spatially restricted species, introgression can progress to the point where the gene pool of the rare species is essentially subsumed into the gene pool of the more common species (Chan et al. 2006; Hegde et al. 2006; Rhymer and Simberloff 1996). Thus, the magnitude of gene flow between montane endemics and their congeners can influence whether or not species-specific adaptive genetic complexes will be maintained over longer time periods.

The southern Appalachians are considered a biodiversity hotspot for terrestrial salamanders of the family Plethodontidae (Duellman and Sweet 1999; Milanovich et al. 2010; Rissler and Smith 2010). The largest Plethodontid genus, *Plethodon*, is divided into two subgroups—a western group of nine species and an eastern group of at least 46 distinct species, some of them cryptic (Wiens et al. 2006). These salamanders have limited dispersal ability (Cabe et al. 2007; Marsh et al. 2008) and may exhibit niche conservatism over evolutionary time (Kozak and Wiens 2010), factors which have likely contributed to the evolution of mountaintop species with very small ranges. For example, the Peaks of Otter salamander (*Plethodon hubrichti*), is restricted to forest habitats above 845 m within a narrow 19 km long stretch of the Blue Ridge Mountains in Virginia (Petranka 1998). The Cheat Mountain salamander, *Plethodon nettingi*, is a federally-threatened species found in a number of small populations on the high Cheat Mountain ridge in West Virginia (Pauley 2007). The Shenandoah salamander, *Plethodon shenandoah*, which is listed as federally endangered, is found in talus outcrops on three mountains in Shenandoah National Park (Highton and Worthington 1967). These species are notable in having among the smallest ranges of any mainland vertebrates in North America (Carpenter et al. 2001; Highton 1995).

Recently, a new endemic mountaintop *Plethodon* was identified in the southern Appalachians. *Plethodon sherando*, the Big Levels salamander, was proposed as a new species on the basis of morphological differences such as longer limbs and fewer trunk vertebrae in comparison to the widespread species *Plethodon cinereus* (Northern Red-Backed salamander) that surrounds it (Highton 2004). In addition, Highton (2004) reported differences in allozyme frequencies between *P. sherando* and *P. cinereus*. Salamanders that are morphologically *P. sherando* have a range

that apparently consists of less than 80 km² along the top of the Big Levels area of Augusta county, Virginia (Highton 2004). *P. sherando* is found along ridgetops and on the slopes of Big Levels down to about 640–670 m (Fig. 1), where populations abruptly transition to animals that appear to be *P. cinereus* (Highton 2004). An additional contact zone is found at high elevations, where Big Levels wraps around to meet the main Blue Ridge (Fig. 1). Little is currently known about *P. sherando* aside from the general outlines of its range and some morphological characters that distinguish it from *P. cinereus*. The species is absent from recent analyses of the phylogeny and evolution of the *Plethodon* genus (e.g. Kozak and Wiens 2006, 2010; Wiens et al. 2006). Moreover, unlike the other mountaintop endemics in the region, *P. sherando* has no special conservation designation, primarily because of its recent discovery and uncertainty about its status.

We used sequence data from two mitochondrial regions and morphological data across two contact zones to examine genetic diversity and differentiation in *P. sherando* and the relationship between this salamander and the widespread species *P. cinereus* that surrounds it. More specifically, we sought to determine: (1) whether animals that are morphologically *P. sherando* versus *P. cinereus* are reciprocally monophyletic, (2) if morphological intermediates between *P. sherando* and *P. cinereus* are found in contact zones at low or high elevation, (3) if morphology and mitochondrial genotype are concordant across individuals in contact zones, and (4) the phylogenetic relationship between *P. sherando* and other salamanders in the *P. cinereus* group.

Methods

The range of *P. sherando* is centered on the Big Levels ridges of Augusta County, VA (type locality W37°55'09", N79°04'00"). Along these ridges, *P. sherando* has been found down to an elevation of about 600 m, though most contact zones with *P. cinereus* appear closer to 660 m. Based on Highton's (2004) collection locations as well as our own surveys, the likely range for *P. sherando* appears to be between 63 km² and 73 km² assuming a lower elevational limit of 600 and 660 m respectively (Fig. 1).

For our study, *P. sherando* and *P. cinereus* samples were collected from two transects in the Big Levels region (Fig. 1). Each transect consisted of a *P. cinereus* reference site, a contact zone containing both species, and a *P. sherando* reference site. Reference sites were identified based on the surveys reported in Highton (2004), whereas contact zones were identified from our own surveys. The first transect covered a typical elevational gradient for these species, running from a *P. cinereus* reference site

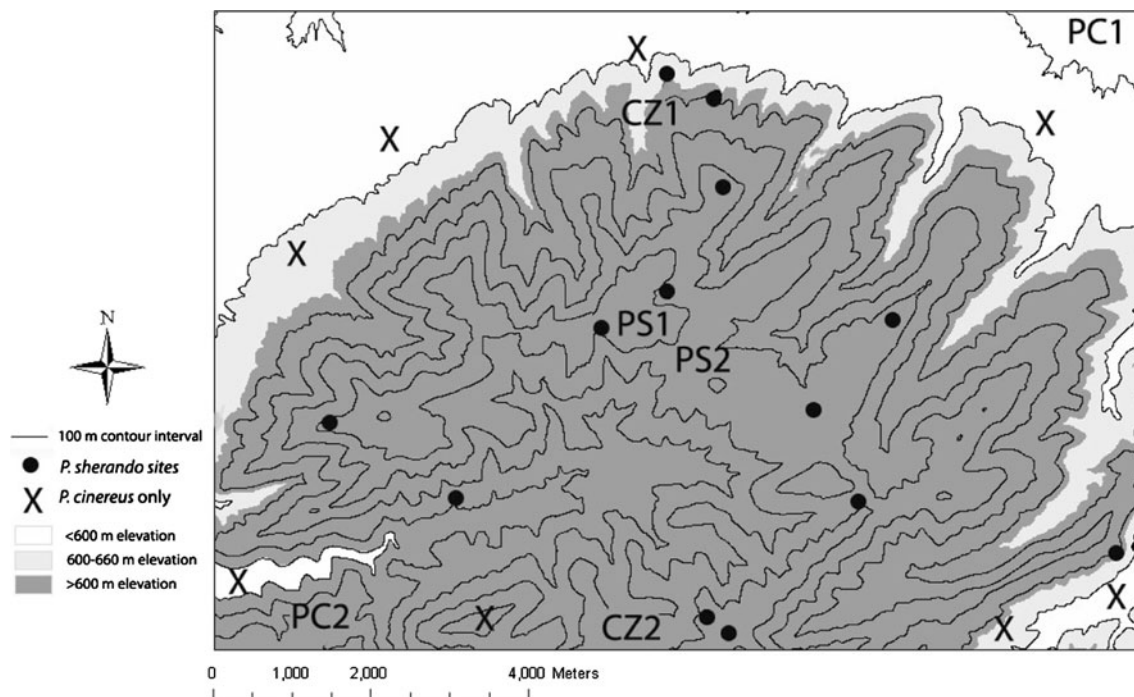


Fig. 1 Map of the range of *P. sherando* and our sampling sites. Filled circles indicate sites where *P. sherando* was found by Highton (2004) or in our own surveys. “X”s indicate sites where *P. cinereus* but not *P. sherando* was found. PS1 and PS2 indicate *P. sherando* reference

sites one and two, PC1 and PC2 indicate *P. cinereus* reference sites, and CZ1 and CZ2 are two contact zone sites where both species were found

(PC1, Fig. 1) at the base of the ridge (580 m) through a mid-elevation (650–670 m) contact zone (CZ1) to a ridgetop *P. sherando* reference site (PS1) at 1,020 m. The second transect remained at a similar elevation (950–1,050 m) for all three sites, running from a *P. cinereus* reference site on the Blue Ridge (PC2), through a contact zone where the Blue Ridge connects to Big Levels (CZ2), and terminating in a *P. sherando* reference site within Big Levels (PS2, Fig. 1). For comparative purposes, we also collected six samples of *P. hubrichti*, the Peaks of Otter salamander from a single site within its mountaintop range approximately 40 km to the southwest. We considered this to be a relevant comparison because *P. hubrichti* has a similar range size to that of *P. sherando*, and it is likewise surrounded by *P. cinereus* at lower elevations.

Collection and morphological measurements

We located salamanders by turning over rocks and logs along the forest floor and searching through the leaf litter. Searches were carried out during the day in April–May and September–October, 2009. When we captured salamanders, we placed them in plastic bags and cooled them before morphological measurements were taken on live animals.

We used morphological features identified in Highton’s (2004) original study for distinguishing *P. sherando* from

P. cinereus. *P. sherando* typically has a wider head, longer limbs, and a shorter trunk than *P. cinereus*. Because *P. sherando* has longer legs and a shorter trunk than *P. cinereus*, the number of intercostal spaces (i.e. the spaces between the ribs, hereafter ICS) when limbs are pressed flat against the body is lower in *P. sherando* than in *P. cinereus*. When corrected for snout-vent length (SVL), this metric shows little or no overlap between taxa (Highton 2004) with *P. sherando* normally having an ICS/SVL ratio <0.13 and *P. cinereus* having a ratio >0.15. Because measurements were taken on live animals, some measurement error was expected. To minimize this error, each measurement was recorded independently by 2–3 different observers and these measurements were averaged for each animal.

After the morphological measurements for each animal were recorded, we collected a 0.5 cm tail tip with forceps from 12 to 16 animals per species per site. Salamanders were then released at the site where they were collected. Plethodontid salamanders lose tails naturally as an antipredator defense (Jaeger and Forester 1993), and tails tips typically regrow within several weeks. Tissue samples from tail tips were stored in collection buffer (10 mM Tris, 10 mM EDTA, pH 8) at 4°C before DNA extraction (Cabe et al. 2007). Each of the 12–16 samples from each site was sequenced for both CO1 and cyt-b.

DNA sequencing

Genomic DNA extraction was performed using the methods described in Connors and Cabe (2003) and Cabe et al. (2007). Genomic DNA was extracted from tissue samples within 24 h of collection using the Promega Wizard® Genomic DNA Purification Kit; (Madison, WI) and quantified using a NanoDrop® spectrophotometer (Wilmington, DE).

We amplified and sequenced segments of the cytochrome oxidase I (COI) and cytochrome B (cyt-b) genes. We chose COI to provide information for the Barcode of Life project (Smith et al. 2008), and we chose cyt-b because this gene has been used frequently in prior phylogenetic analyses of the *Plethodon* genus (Wiens et al. 2006).

PCR was used to amplify a 750 bp fragment of the COI using LepF1-T3 (a T3 tailed version of the LepF1 primer, AATTAACCCTCACTAAAGATTCAACCAATCATAAAGATATTGG') and LepR1 (5'-TAAACTTCTG GATGT CCAAAAATCA-3') or LepF1 (ATTCAACCAATCATAAAGATATTGG) and LepR1-T7 (TAATACGACTCACTATAGGGTAAACTTCTGGATGTCCAAAAATCA) following Hebert et al. (2004) and Smith et al. (2008). COI PCR reactions (50 µl) contained 1× GoTaq Buffer®, 1.5 mM MgCl₂, 200 µM dNTP's, 0.5 µM LepF1-T3, 0.5 µM Lep-R1, 1.25 U/50 µl *Taq* Polymerase, and 0.02–0.4 ng/µl genomic DNA as template, using the GoTaq® PCR Core kit (Promega, Madison, WI). Cycling conditions were 94°C for 5 min followed by 34 cycles of 94°C for 30 s, 53.5°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 5 min.

A portion of the cytochrome B gene was amplified using a T3 tailed primer PcCytB-F-T3 (5'-AATTA A CCCTCACTAAAGGGCTCAACCAAAACCTTTGAC C-3') and PcCytB-R (5'-TAGCCCCCAATTTTGGTTT ACA-3'), both of which were designed using the complete *P. cinereus* mtDNA sequence available in GenBank (accession number AY728232.1). Cyt-b reactions mirrored COI reactions, except that primers annealed at 47°C for 45 s. Products from successful PCR reactions were purified using the Wizard SV Gel and PCR Clean up System (Promega, Madison, WI). Sequencing reactions for both genes were generated using an IR700 labeled T3 primer and the SequiTherm Excel® II Sequencing Kit-LC (Epicenter, Madison, WI). The PCR products were sequenced using a LiCor 4300 DNA analyzer and base calls automatically scored with eSeq v. 3.1 (LiCor, Lincoln, NE). A subset of COI sequences was validated by sequencing with an IR800 labeled T7 primer. Sequences contributing to our analysis are archived in GenBank as samples JF731278–JF31333.

Sequence alignment, distance, and diversity

Sequences for each gene were manually edited, trimmed, and aligned using ClustalW in MEGA 4 (Tamura et al. 2007). Alignments were unambiguous (i.e. no gaps were required) and resulted in a 684 bp segment of cyt-b and a 599 bp segment of COI. Some sequences did contain ambiguous bases, particularly near the ends of the sequences. Ambiguous bases were treated as missing data in all analyses and unique haplotypes were not classified based on ambiguous bases. In principle, ambiguities can be caused by mixtures of mitochondrial genes and nuclear copies (Bensasson et al. 2001). However, in 26 samples that were sequenced in both directions the same ambiguities were never detected. Furthermore, no stop codons or in-dels were found when individual sequences were translated, suggesting that we successfully amplified mtDNA rather than nuclear copies of mitochondrial sequences. The two segments were concatenated for the analyses below (1,283 bp) unless otherwise specified.

We used the program DnaSP v5 (Rozas et al. 2003) to calculate measures of mtDNA sequence diversity. For the full set of putative *P. sherando* samples, we calculated haplotype diversity (*h*, the probability that two randomly chosen haplotypes differ) and nucleotide diversity (π , average number of pairwise differences per nucleotide site). We also calculated these measures for haplotypes sampled exclusively from the two contact zones for both *P. sherando* and *P. cinereus*. This allowed us to compare sequence diversity between the two lineages over the same spatial scale.

We compared potential models of nucleotide substitution using jModeltest 0.1 (Posada 2008). For both genes, model selection based on AIC yielded a generalized time reversible model with rate variation among sites with a model weight >0.90 (GTR + Γ , model-averaged $\Gamma = 0.78$). We used this model in analyses of genetic parameters, phylogenetic reconstruction, and time to most recent common ancestor (see below).

Phylogenetic reconstruction

We estimated relationships among haplotypes using maximum likelihood (ML) and Bayesian methods, implemented in PHYLIP 3.69 (Felsenstein 1993) and Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), respectively. For computational efficiency, these analyses were carried out on a subset of 24 samples that included at least two samples from each lineage at each site (two contact zone sites plus two reference sites for both *P. sherando* and *P. cinereus*).

We first estimated relationships among concatenated *cyt-b* and *CO1* haplotypes. We used genbank sequences for *Plethodon petraeus* (the Pigeon Mountain salamander; accession numbers AY728222.1, NC_006334.1) as an outgroup. Because *CO1* sequences are not available for most *Plethodon*, we also estimated relationships among *cyt-b* haplotypes for an expanded set of species, including *P. shenandoah* (the Shenandoah salamander; AY378043.1, AY378044.1, AY378045.1, AY378046.1), and *Plethodon serratus* (the Southern Red-Backed salamander; DQ994981.1, DQ994982.1).

For maximum likelihood analyses, we used four gamma rate categories and a search strategy that included iterations of branch length in all topologies. Five hundred bootstrap replicates were used to test the support for each node. For the Bayesian analyses, we used 1,000,000 MCMC generations with samples taken every 100 time steps. The first 25% of the parameter estimates and trees were discarded as burn-in. Four independent runs from different random starting trees produced nearly identical parameter estimates, and in all four runs the log-likelihood plot had stabilized by 250,000 generations.

Time to most recent common ancestor

Time to the most recent common ancestor (MRCA) for *P. sherando* and *P. cinereus* was estimated for unique haplotypes using a coalescent-based approach in BEAST 1.5.4. (Drummond and Rambaut 2007). We used a strict molecular clock model (Tajima's relative rates test, $P = 0.19$). We took mean and standard deviation rates of evolution from the fossil-calibrated plethodontid phylogeny of Mueller (2006): 1.04 (± 0.27) substitutions per 100 million years for *CO1* and 0.62 (± 0.16) substitutions per 100 million years for *cyt-b*. MCMC parameters were set to a chain length of 20 million with a burn-in of 2 million and parameters logged every 1,000 trees. Runs from four different sets of random initial parameter values yielded the same distribution for time to MRCA, suggesting that convergence had been achieved.

Morphological analysis

We conducted several analyses of morphology to examine the distribution of phenotypes across contact zones and reference sites. First, we used cluster analysis to identify the optimal number of clusters based on morphological measurements (interspaces per unit snout-vent length and head width per unit snout-vent length). Clustering was conducted separately for *P. sherando* and *P. cinereus* reference sites and for the contact zones between the two species. To assess the validity of grouping salamanders into two species, we compared the fit of a two-cluster model to

the fit of a single cluster model or a three cluster model. Fits were compared using Bayesian Information Criteria (BIC) in the R program Mclust 3.0 (Fraley and Raftery 2006).

Following this initial cluster analysis, we used linear discriminant function analysis (LDFA) in SPSS v. 17.0 (SPSS Inc., Chicago, IL) to determine the extent to which salamanders from the *P. sherando* and *P. cinereus* reference sites were morphologically distinct from one another. We created a linear discriminant function for location (*P. sherando* or *P. cinereus* reference site) based on 2 predictor variables—intercostal spaces per unit snout-vent length, head width per unit SVL. We then used “leave-one-out” cross validation (Hastie et al. 2001) to assess the performance of the discriminant function. In this procedure, each point is left out of the model, then reassigned to a location (*P. sherando* or *P. cinereus* site) based on its morphology. These assignments can then be compared to the true origin of the animal in order to assess the ability of the model to discriminate animals from the different classes of sites.

We carried out an analogous LDFA on contact zone samples for which we also had mtDNA sequences. Samples were assigned to species based on the morphological variables given above. These species assignments were then validated by comparing them to the lineage to which their mtDNA had been assigned. If there is little introgression between the lineages and their morphology is distinct, assignment accuracy should be close to 100%. Alternatively, extensive introgression between lineages would be expected to produce some individuals with morphology that is non-concordant with mtDNA genotype.

We additionally carried out a two-way analysis of variance on continuous morphological characters (interspaces/SVL and head width/SVL) for both contact zone and reference site samples. Independent variables were site type (reference or contact zone) and mtDNA lineage (*P. sherando* or *P. cinereus*). An interaction between site type and lineage would indicate that morphological differences between mtDNA lineages in contact zones differed in magnitude from those differences at reference sites. If morphological differences were less pronounced at contact zones, this would suggest introgression between lineages.

Population history

We examined the genetic structure of *P. cinereus* in contact zones versus reference sites and ask whether contact zones might represent areas where *P. cinereus* have recently infringed on the range of *P. sherando*. In general, in areas of recent expansion, one would expect lower genetic diversity, an excess of rare haplotypes given the level of nucleotide diversity (i.e., negative values for F_u 's F_s), and a unimodal mismatch distribution (Harpending 1994;

Johnson et al. 2007; Schneider and Excoffier 1999). We examined these values separately for *P. cinereus* in each of the two contact zones (CZ1 and CZ2). Haplotype diversity in *P. sherando* was not high enough to permit analogous tests in this species.

Results

Plethodon sherando samples exhibited very low genetic diversity as compared to *P. cinereus* samples (Table 1). Across sites with no ambiguous bases, we recovered just 3 haplotypes with 2 variable nucleotide sites for *P. sherando* at both loci. Diversity was correspondingly low ($h = 0.41$, $\pi = 0.0009$). *P. sherando* samples from just the two contact zones yielded similar estimates of diversity ($h = 0.56$, $\pi = 0.0010$). In contrast, *P. cinereus* showed an order of magnitude greater nucleotide diversity within the same two contact zones ($h = 0.633$, $\pi = 0.0096$, with 5 haplotype and 14 variable sites (Table 1). Our six samples of *P. hubrichti* were intermediate between *P. sherando* and *P. cinereus* in sequence diversity ($h = 0.60$, $\pi = 0.0051$).

Genetic distance

Plethodon sherando COI and cyt-b haplotypes were highly divergent from *P. cinereus* haplotypes. Including only sites with no ambiguous bases, average pairwise percent sequence divergence was 16.7%, and genetic distance (GTR + Γ) between the lineages was 0.27 (± 0.05). For comparison, the genetic distance between *P. cinereus* and *P. hubrichti* was 0.18 (± 0.04).

Phylogenetic analysis

Phylogenies for both mtDNA segments based on maximum likelihood and Bayesian methods yielded identical topologies (Fig. 2). Reciprocal monophyly of animals that were morphologically *P. sherando* or *P. cinereus* was strongly supported (100% bootstrap support and 100% posterior probability for each clade). *P. cinereus* samples grouped into two clusters—one that corresponded with the high elevation sites (PC2 and CZ2) and one that corresponded to the low elevation sites (PC1 and CZ1). Because *P. sherando* showed very little sequence divergence across the range, there was no clustering based on collection site. Interestingly, *P. cinereus* grouped with *P. hubrichti* (rather than *P. sherando*), suggesting that *P. sherando* is a more distantly related lineage within the *P. cinereus* group.

In the expanded cyt-b phylogeny (Fig. 3), *P. sherando* was sister to the *P. serratus* rather than the *P. cinereus*. Although support for this node was only moderate (bootstrap probability = 0.78, posterior probability = 0.90), Highton (2004) reported the same putative relationship based on allozyme data. In any case, the phylogeny suggests that *P. sherando* is more distantly related to *P. cinereus* than are any of the other mountaintop species in the region (*P. shenandoah*, *P. hubrichti*).

Time to most recent common ancestor

Plethodon sherando and *Plethodon cinereus* shared a common ancestor about 11.4 million years ago based on combined estimation from cyt-b and COI (95% HPD 9.0–13.8). *P. sherando* and *P. serratus* were estimated to

Table 1 Genetic diversity for different species and sets of sampling locations

Species	Sites	<i>n</i>	Haplotypes	Variable sites	<i>h</i>	π	F_s
<i>P. sherando</i>	All	62	3	2	0.41	0.0009	NA
<i>P. sherando</i>	Contact zones	31	3	2	0.56	0.0010	NA
<i>P. sherando</i>	CZ1	15	3	2	0.26	0.0003	NA
<i>P. sherando</i>	CZ2	16	2	1	0.13	0.0002	NA
<i>P. sherando</i>	PS1	15	3	2	0.53	0.0006	NA
<i>P. sherando</i>	PS2	16	2	2	0.15	0.0005	NA
<i>P. cinereus</i>	All	55	6	16	0.65	0.0082	NA
<i>P. cinereus</i>	Contact zones	25	5	14	0.63	0.0096	NA
<i>P. cinereus</i>	CZ1	13	3	4	0.30	0.0011	−0.17
<i>P. cinereus</i>	CZ2	12	3	2	0.32	0.0006	−1.33*
<i>P. cinereus</i>	PC1	15	2	1	0.13	0.0002	−0.65
<i>P. cinereus</i>	PC2	15	4	3	0.70	0.0015	−0.53
<i>P. hubrichti</i>	All ^a	6	4	10	0.90	0.0089	NA

“*h*” is the probability that two randomly chosen haplotypes are not identical, “ π ” is the mean number of differences per site between randomly chosen sequences, and “ F_s ” is Fu’s *F*, which measures the proportion of rare haplotypes

* $P < 0.05$

^a Six samples from *P. hubrichti* at one site near the eastern end of their range

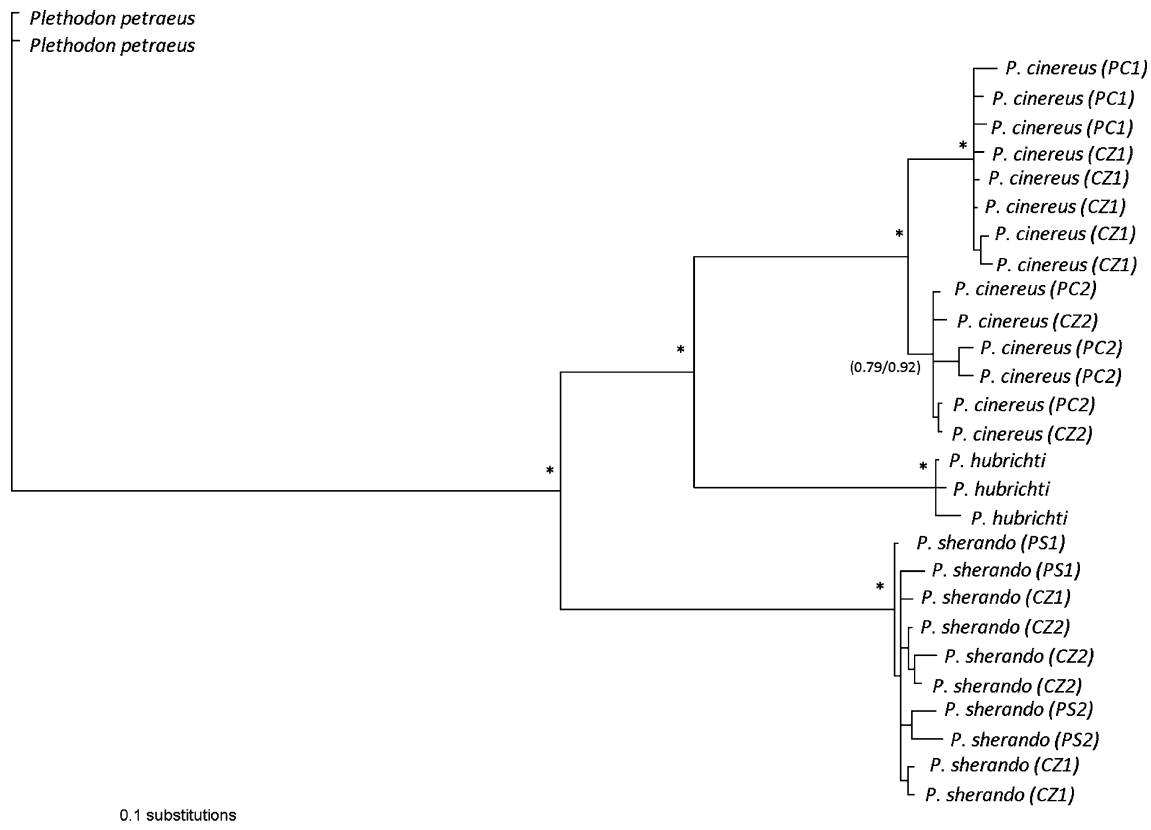


Fig. 2 Maximum likelihood and Bayesian phylogeny based on concatenated cyt-b and CO1 sequences with *P. petraeus* used as an outgroup. Numbers in parentheses are bootstrap support (proportion

of bootstrap replicates) and posterior probabilities for each node. Nodes with support >0.98 are indicated with an “asterisk”. Site locations for samples (see Fig. 1) are given for each sample

have shared a common ancestor 6.0 million years (95% HDP 4.4–7.6) based on cyt-b sequences. Given the position of *P. sherando* on the phylogeny reported above (i.e. sister to *P. serratus*), these estimates are consistent with the fossil-calibrated phylogeny of Wiens et al. (2006).

Morphological analysis

Cluster analysis identified two clusters based on morphology for both reference site salamanders and for contact zone salamanders. For reference site samples ($n = 109$), BIC was substantially lower for a two-cluster model (equal volume, equal shape) as compared to a three-cluster model ($\Delta BIC = 14.1$) or a single cluster ($\Delta BIC = 85.0$). For contact zone samples ($n = 79$), BIC was also lowest for a two-cluster model as compared to three clusters ($\Delta BIC = 5.7$) or a single cluster ($\Delta BIC = 39.9$).

LDFA showed morphology at reference sites to be highly distinct between *P. sherando* and *P. cinereus*. Leave-one-out cross validation at reference sites yielded 100% classification success by species/site. Contact zone samples were, for the most part, also distinct. When individuals were classified based on morphology and

validated based on mtDNA lineage, classification success was 98% for each lineage (Fig. 4). One *P. sherando* haplotype was classified as a morphological *P. cinereus* and one *P. cinereus* haplotype was classified as a morphological *P. sherando*. These 2 individuals were possibly hybrids. However, both individuals were juveniles, and lower posterior probabilities for other juveniles in the sample suggested that the classification model may have been less reliable for smaller salamanders. ANOVA indicated that, overall, salamanders in the contact zone were at least as distinct as salamanders at the reference sites. Interactions between lineage (*P. sherando* or *P. cinereus*) and site type (reference site or contact zone) were non-significant for both intercostal spaces/SVL ($F_{1,109} = 0.69$, $P = 0.41$) and for head width/SVL ($F = 2.97$, $P = 0.08$). The marginal result for head width ($P = 0.08$) is in the opposite direction than would be predicted with hybridization—lineages in the contact zone were more different than were lineages at the reference sites (Fig. 5). This result is plausibly consistent with findings of character displacement in head shape at contact zones between other pairs *Plethodon* (e.g. Adams 2004; Adams et al. 2007). Increased divergence in head width in contact zones was

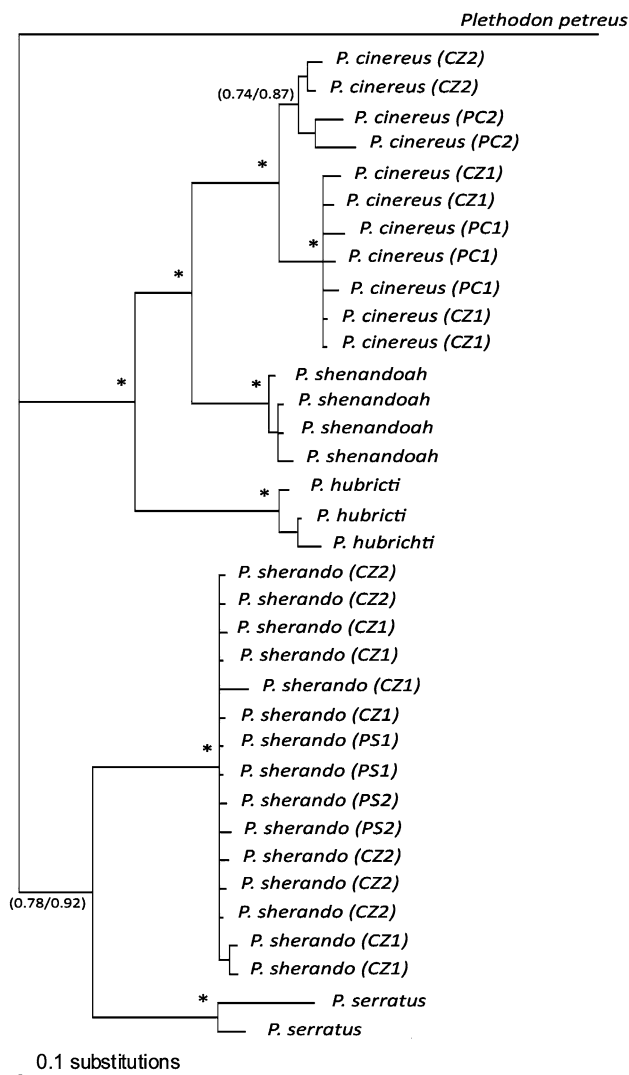


Fig. 3 Maximum likelihood and Bayesian phylogeny for cyt-b with *P. petraeus* used as an outgroup. Numbers in parentheses are bootstrap support (proportion of bootstrap replicates) and posterior probabilities for each node. Nodes with support >0.98 are indicated with an “asterisk”. Site locations for samples (see Fig. 1) are given for each sample. *P. serratus* and *P. shenandoah* samples are taken from GenBank

in fact statistically significant ($F_{1,219} = 4.74$, $P = 0.03$) when we increased our sample size by classifying salamanders by intercostal spaces instead of by mtDNA lineage.

Population history

In *P. cinereus*, sequence diversity was comparable between contact zone sites and reference sites (Table 1). Fu’s F_s was significantly negative in CZ2 ($F_s = -1.32$, $P = 0.007$), which is consistent with recent population expansion, though it was not significantly negative in CZ1

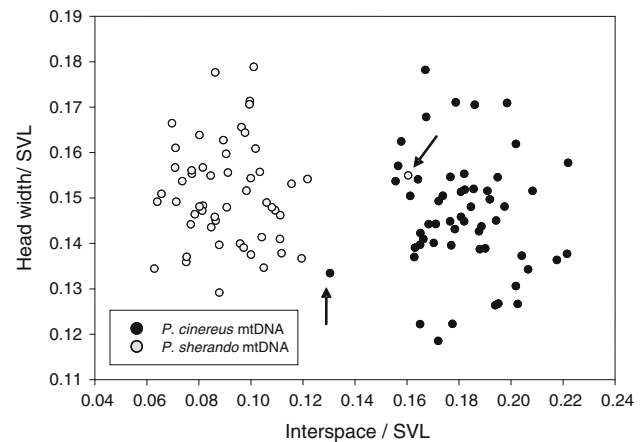


Fig. 4 Results for classification of contact zone salamanders with known mtDNA lineages based on morphological characters. All morphological classifications were consistent with mtDNA lineages except for the two indicated with arrows

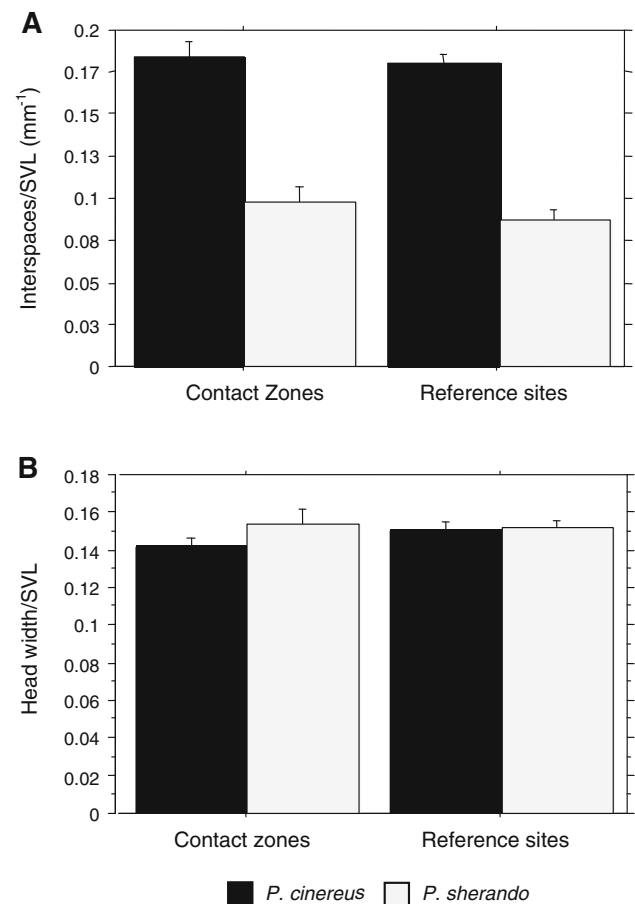


Fig. 5 Comparison of morphological differences between salamander species (as determined by mtDNA lineage) in contact zone areas versus reference sites. Tests for interactions between species and site type were used to ask whether salamanders were more similar in contact zones as compared to reference sites, as would occur with substantial introgression. Results are shown for: **a** intercostal spaces per unit SVL; **b** head width per unit SVL

($F_s = -0.17$, $P = 0.079$). F_s was not significantly different from zero for either of the reference sites PC1 or PC2 ($F_s = -0.65$, $P = 0.11$, and $F_s = -0.53$, $P = 0.23$). Harpending's raggedness index, which indicates departure from unimodality in the mismatch distribution, only approached significance for the reference site PC2 ($P = 0.07$). The index was not significantly different from zero at any of the other three sites, indicating that a recent expansion model could not be rejected for these sites.

Discussion

Our results highlight the genetic uniqueness of the *P. sherando* lineage and the need to consider this species in conservation plans for the region. We found that morphological *P. sherando* were highly distinct from *P. cinereus* in their mtDNA sequences, with 17% sequence divergence across two mitochondrial regions. Our analysis of morphology in contact zones and reference sites further supported the distinction between these two taxa; all but two individuals were correctly assigned as *P. sherando* or *P. cinereus* on the basis of a few morphological characters, and there was little overall evidence of morphological intermediates detected in contact zones. The general concordance between mitochondrial genotype and morphology suggests that there has been at most a small amount of introgression between the lineages where they live in sympatry. Our phylogenetic analyses further supported this conclusion; *P. sherando* and *P. cinereus* morphotypes were reciprocally monophyletic using both maximum likelihood and Bayesian methods. These findings, combined with the very low genetic diversity found in *P. sherando*, suggest that *P. sherando* has a distinct evolutionary history and has probably undergone some period of isolation on or near the Big Levels ridgetop.

Conservation issues

Plethodon sherando's extremely limited distribution (<80 km²) and its abrupt replacement by *P. cinereus* along its range boundaries create several potential conservation issues for this species. In general, anytime a species with a restricted distribution is surrounded by a more common congener, genetic swamping via introgression may be considered as a potential threat (Chan et al. 2006; Rhymer and Simberloff 1996). Moreover, many other *Plethodon* species are known to hybridize (Highton 1998, 1999) and hybridization may lead to substantial introgression in these species (Wiens et al. 2006; Weisrock et al. 2005). Indeed, we did find two misclassified individuals within the contact zone; these misclassifications could be due to measurement error, but they could represent true hybrids between

P. sherando and *P. cinereus*. However, given the small number of these potential hybrids and the lack of any misclassifications at reference sites, we suggest that the threat of "genetic swamping" by *P. cinereus* does not appear to be an immediate threat to the persistence of *P. sherando*. Further studies at other contact zones between these two species would be helpful to ensure that introgression is not occurring extensively at other sites within *P. sherando*'s restricted range.

One of the most critical threats to mountaintop endemics like *P. sherando* may be climate change coupled with range expansion by competitors (Gilman et al. 2010). Milanovich et al. (2010) modeled salamander persistence in the southern Appalachians as a function of climate variables and showed that climate warming could lead to major range contractions. Their study did not include salamanders of the *P. cinereus* group; however, this group is also known to be sensitive to climate. For instance, Arif et al. (2007) found that the lower limit of *P. hubrichti*'s range appeared to be set by climate. For many mountaintop species, replacement by competing species with higher thermal tolerances is potentially a greater threat than just a change in the distribution of suitable habitats (Gilman et al. 2010). If *P. cinereus* outcompetes *P. sherando* at higher temperatures, expansion of *P. cinereus* into *P. sherando*'s range would likely follow climate warming. That said, the interaction of *P. sherando* with *P. cinereus* does not appear to reflect the traditional scenario of a mountaintop endemic surrounded by a lowland adapted species. Southwestern Virginia is near the southern limit of *P. cinereus*' range, and *P. cinereus*' complete range includes both high elevations within the Blue Ridge and colder regions as far north as central Quebec (Petranka 1998). Thus, it is not clear that warmer conditions would favor *P. cinereus* over *P. sherando*.

Evidence that *P. cinereus* is already expanding into the range of *P. sherando* was mixed. One contact zone site had a significantly negative value for Fu's F_s (which can be indicative of recent population expansion) and one reference site had a value for Harpending's raggedness index that approach significance (i.e. approached rejection of an expansion model). However, both reference sites and contact zone sites had similar within-site genetic diversity, and most tests for expansion were non-significant. Ultimately, larger sample sizes will be necessary to understand the recent history of these contact zone populations.

Phylogeography

The phylogenetic analysis of *P. sherando* is interesting in that it suggests that, unlike *P. shenandoah*, *P. sherando* is not a sister species to *P. cinereus*. In fact, our analysis of cyt-b sequences suggests a potential affiliation between

P. sherando and the *P. serratus* (Fig. 3). Although statistical support for this node was only moderate (79% ML bootstrap support and 92% Bayesian posterior probability), Highton (2004) suggested a similar relationship based on independent allozyme data, and more recent nuclear data appear to confirm this affiliation (Highton, personal communication). *P. serratus*' current range includes parts of Arkansas, Missouri, North Carolina, Alabama, Georgia, Louisiana and Oklahoma (Petranka 1998). At the North-eastern limit of its range, *P. serratus* is only several hundred kilometers from the range of *P. sherando*. Given a divergence time of about 6 million years between the two lineages, it would not be surprising if *P. sherando* represents a lineage that became isolated in the Blue Ridge as the range of a *P. serratus* ancestor shifted southwards.

Study limitations

Our study has several important limitations. Most notably, we used concordance between mitochondrial genotypes and morphology to investigate the potential hybridization of the *P. sherando* and *P. cinereus* lineages. Although this approach has been used elsewhere (Babik et al. 2005; Carpenter et al. 2001; Szymura et al. 2000), clearly nuclear markers would have provided more direct evidence for or against hybridization. A second limitation is that we collected samples from only two contact zones, one at the lower limit of *P. sherando*'s elevational range and one near the upper limit. While we found no morphological evidence for hybridization at these sites, it is possible that hybridization was occurring at other sites where we did not collect. For example, morphological and allozymic data suggest hybridization between *P. shenandoah* and *P. cinereus* at one site but not at two others (Highton and Worthington 1967; Highton 1999). A third limitation is that we sampled only a portion of *P. sherando*'s range. Although we sampled opposite ends of the range, it is possible that we missed pockets of genetic diversity that would have changed our understanding of the *P. sherando* lineage. Finally, with only two mitochondrial regions, gene trees could differ substantially from true species trees (Degnan and Rosenberg 2009). That said, our results agree with the results of prior studies based on allozymes (Highton 2004) and forthcoming phylogenies of the *Plethodon* genus based on a large number of nuclear and mitochondrial markers (R. Highton, personal communication).

Conservation status of *Plethodon sherando*

Plethodon sherando currently lacks any special status within the U.S. or the State of Virginia because of its

recent recognition and a lack of knowledge of its biology. In contrast, the other four mountaintop salamanders in the region all have some formal conservation status. The Shenandoah salamander (*P. shenandoah*) is listed as Federally Endangered and thus protected by the U.S. Endangered Species Act. The Cheat Mountain salamander (*P. nettingi*) is Federally Threatened and covered by a formal recovery plan. Both the Cow Knob salamander (*P. punctatus*) and the Peaks of Otter salamander (*P. hubrichti*) are listed federally as a “species at risk,” and are state-listed as a “species of concern.” For *P. punctatus*, this status means that most of its high-elevation habitat within National Forest is protected from logging (United States Forest Service 2011). For *P. hubrichti*, a primary habitat area is protected from logging and a secondary habitat area requires the consideration of management impacts on the species. Timber harvest within the secondary range of *P. hubrichti* is scheduled outside the normal surface activity periods for the species, and shelterwood and coarse woody debris are retained at sites where harvest occurs. Based on our findings, we suggest that *P. sherando* should merit a similar status to that of *P. hubrichti*. The range sizes of the two species are similar ($\sim 100 \text{ km}^2$ for *P. hubrichti* versus $\sim 80 \text{ km}^2$ for *P. sherando*), and both species have ranges that consist primarily of high-elevation National Forest land. Moreover, both species' distributions are entirely surrounded by *P. cinereus* at lower elevations, suggesting that competition, climate change, forest degradation, and interactions between these factors may be the greatest threats to long-term persistence. Based on these considerations, we believe that forest planning for the region should recognize the conservation needs of *P. sherando* as a spatially-restricted endemic species. This recognition could include the designation of *P. sherando* core habitat as a habitat conservation area, analogous to the habitat conservation areas that exist for both *P. hubrichti* and *P. punctatus*. In addition, as with *P. hubrichti* and *P. punctatus*, continued monitoring of range limits and restrictions on timber harvest within the species' range should be important components of a long-term conservation strategy.

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