Low Genetic Diversity and High Interspecific Divergence in a Mountaintop Salamander with an Extremely Limited Range

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Abstract The Southern Appalachians are a biodiversity hotspot for salamanders, and several montane endemics occur in the region. Here, we present the first genetic data for *Plethodon sherando*, a terrestrial salamander recently discovered along a single ridgetop in the Blue Ridge of Virginia. We sequenced two mitochondrial loci (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 Northern Red-Backed salamanders, *Plethodon cinereus*. We found *P. sherando* and *P. cinereus* sequences to be reciprocally monophyletic and highly divergent (~17%). *P. sherando* exhibited very low sequence diversity (π = 0.0010) as compared to *P. cinereus* from the same locations (π = 0.0096) and as compared to *P. hubrichti* (π = 0.0051), another nearby mountaintop endemic. Salamander morphology in the contact zone was at least as distinct as morphology at reference sites, and linear 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 Southern Red-Backed salamanders, *P. serratus*, rather than *P. cinereus* or any of the 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 very limited range (< 80 km^2), we believe that *P. sherando* should merit a conservation status similar to that of other mountaintop salamanders in the region.

Keywords *Plethodon sherando*· Big Levels Salamander · mtDNA · phylogeography · population genetics · conservation

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 have also been identified as important factors in the creation of biodiversity, with mountain ranges providing opportunities for allopatric speciation (Knowles 2001; Kozak and Wiens 2006; Xu et al 2010). This may be particularly true in the tropics, where changes in elevation have been shown to correlate with very different species assemblages (e.g. Lovett 1998; Terborgh 1977; Vasudevan 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).

Unfortunately, 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 (La Sorte and Jetz 2010; Milanovich et al. 2010). And for many montane species, replacement by competing species with 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 also be susceptible to genetic swamping via introgression. Gene flow at the boundaries of two related species is relatively common and does not necessarily threaten either species (Colliard 2010; Mallet 2005; Mallet et al. 2007). However, in the cases of rare or spatially restricted species, introgression can progress to the point where the rarer species is genetically swamped by its more common counterpart (Chan et al. 2006; Hegde et al. 2006; Rhymer and Simberloff 1996). Therefore, understanding gene flow between montane endemics and their congeners is highly relevant for their conservation.

The Southern Appalachians is considered a biodiversity hotspot for terrestrial salamanders of the family Plethodontidae (Duellman and Sweet 1999; Milanovich et al. 2010, Rissler and Smith 2010). 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 Shendandoah 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. The Big Levels salamander, *Plethodon sherando*, was proposed as a new species of *Plethodon* on the basis of morphological and allozymic differences between *P. sherando* and the widespread Northern Red-Backed salamanders (*P. cinereus*) that surrounds it (Highton 2004). The Big Levels salamander has a range that apparently consists of less than 80 km^2 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, where populations rapidly transition to animals that appear to be *P. cinereus* (Fig 1). 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 group (e.g. Kozak and Wiens 2006, 2010; Wiens et

al. 2006). Moreover, unlike the other mountaintop endemics in the region, the Big Levels salamander has no special conservation status, primarily because of its recent discovery and uncertainty about its status.

We used sequence data from two mitochondrial loci and morphological data across two contact zones to examine genetic diversity and differentiation in the Big Levels salamander and the relationship between this salamander and the widespread Northern Red-Backed salamander that surrounds it. More specifically, we sought to determine: 1) whether *P. sherando* and *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. We interpret the results for each of these issues in the context of the phylogeography and conservation of the Big Levels Salamander in its native habitat.

Methods

Species

The Big Levels salamander, *Plethodon sherando*, and the Red-Backed salamander, *Plethodon cinereus* belong to the woodland salamander genus, *Plethodon*. This genus is divided into two subgroups, a western group of nine species and an eastern group of at least 46 distinct species (Wiens et al. 2006). Members of this genus are terrestrial, lay their eggs on land, and are capable of living their entire lives without an aquatic habitat (Highton 1995). Many of the eastern species of the *Plethodon* genus are morphologically cryptic are identified primarily by their genetic uniqueness (Highton 1995).

P. sherando is a semi-cryptic species with many morphological traits similar to those of *P. cinereus* (Highton 2004). *P. sherando* was proposed as a new species of *Plethodon* in 2004 on the basis of morphological and allozymic differences between *P. sherando* and *P. cinereus* (Highton 2004). *P. sherando* inhabits a known range of less than 80 km^2 along the top of the Big Levels ridge within the Blue Ridge Mountains in Virginia (Highton 2004). *P. sherando*'s range is surrounded at lower elevations and

at all sides by *P. cinereus* (Fig 1). We included Highton's (2004) collection locations as well as our own locations in a GIS database, and projected a likely range for *P. sherando* as between 63 km^2 and 73 km^2 based on a lower elevational limit of 600 m (minimum observed) or 660 m (most commonly observed).

Study site

P. sherando and *P. cinereus* samples were collected from two transects in the Big Levels region of Augusta County, Virginia (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 at the base of the ridge (580 m) through a mid-elevation contact zone (650-670 m) to a ridgetop *P. sherando* reference site (1020 m). The second transect remained at a similar elevation (950-1050 m) for all three sites, running from a *P. cinereus* reference site on the Blue Ridge, through a contact zone where the Blue Ridge connects to Big Levels, and terminating in a *P. sherando* reference site within Big Levels (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.

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 both spring and fall salamander activity periods. When we captured salamanders, we placed them in plastic bags and cooled them before morphological measurements were taken on live animals.

For the most part, we used morphological features identified in Highton's (2004) original study for distinguishing *P. sherando* from *P. cinereus. P. sherando* typically has a slightly wider head, longer limbs, and a shorter trunk than *P. cinereus*. Because *P. sherando* has a shorter trunk and longer legs 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*. This metric shows little or no overlap between taxa (Highton 2004), and we used it to make preliminary classifications for animals in the field. Additionally, we noticed that *P. sherando* (but not *P. cinereus*) sometimes had a brassy flecking covering the red-stripe and dorsum. This may be a characteristic of particular *Plethodon* populations rather than species since *P. cinereus* has similar flecking at other sites in its range (R. Highton personal communication). However, near contact zones, the flecking did appear as a useful character; therefore, we included it along with head width, the number of intercostal spaces, and snout vent length as our primary morphological measurements. Because measurements were taken on live animals, some measurement error was expected. To minimize the influence of this error, each measurement was recorded independently by 2-3 different observers. Continuous measurements (intercostal spaces, head width, and SVL) were then averaged for each animal. For the presence of brassy flecking, only animals reported to have this trait by all observers were recorded as having the trait.

After the morphological measurements for each animal were recorded, we collected a 0.5 cm tail tip with forceps from up to 12-14 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 Forrester 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 for up to 24 hours before DNA extraction (Cabe et al. 2007).

DNA Sequencing

Genomic DNA extraction was performed using the method outlined in Connors and Cabe (2003) and Cabe et al. (2007). Genomic DNA was extracted from tissue samples within 24 hours of collection using the Promega Wizard® Genomic DNA Purification Kit; (Madison, WI) and quantified using a NanoDrop® spectrophotometer (Wilmington, DE). Extracted DNA was stored at -20°C.

We amplified and sequenced segments of the cytochrome oxidase I (CO1) and cytochrome B (cyt-b) genes. We chose CO1 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 CO1 using LepF1-T3 (a T3 tailed version of the LepF1 primer, AATTAACCCTCACTAAAGATTCAACCAATCATAAAGATATTGG') and LepR1 (5'-TAAACTTCTG GATGTCCAAAAAATCA-3') or LepF1 (ATTCAACCAATCATAAAGATATTGG) and LepR1-T7 (TAATACGACTCACTATAGGGTAAACTTCTGGATGTCCAAAAAATCA) following Hebert et. al (2004) and Smith et a. (2008). CO1 PCR reactions (50 µl) contained 1X GoTaq Buffer®, 1.5 mM $MgCl_2$, 200µM dNTP's, 0.5 µM LepF1-T3, 0.5 µM Lep-R1, 1.25u/50µl *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 five min.

A portion of the cytochrome B gene was amplified using a T3 tailed primer PcCytB-F-T3 (5'-AATTAA CCCTCACTAAAGGGCTCAACCAAAACCTTTGACC-3') and PcCytB-R (5'-TAGCCCCCAATTTTGGTTTACA-3'), both of which were designed using the complete *P. cinereus* mtDNA sequence available in GenBank (accession number AY728232.1). Cyt-b reactions mirrored CO1 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). The PCR products were sequenced using a LiCor 4300 DNA analyzer and base calls automatically scored with eSeq v. 3.1 (LiCor, Lincoln, NE). Sequencing reactions for both genes were generated using an IR700 labeled T3 primer and the SequiTherm Excel® II Sequencing Kit-LC (Epicenter, Madison, WI). A subset of CO1 sequences was validated by sequencing with an IR800 labeled T7 primer. Sequences contributing to our analysis are archived in GenBank as samples JF731278-JF31333.

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169 Sequence alignment, distance, and diversity

170 Sequences for each gene were manually edited, trimmed, and aligned using ClustalW in MEGA 4
171 (Tamura et al. 2007). Alignments were unambiguous (i.e. no gaps were required) and resulted in a 684 bp
172 segment of cytB and a 599 bp segment of CO1. No stop codons or in-dels were found when individual
173 sequences were translated, suggesting that we successfully amplified mtDNA rather than nuclear copies
174 of mitochondrial sequences. The two segments were concatenated for the analyses below (1283 bp)
175 unless otherwise specified.

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

183 We compared potential models of nucleotide substitution using jModeltest 0.1 (Posada et al.
184 2008). For both genes, model selection based on AIC yielded a generalized time reversible model with
185 rate variation among sites with a model weight > 0.90 (GTR + Γ, model-averaged $\Gamma = 0.78$). We used
186 this model in analyses of genetic parameters, phylogenetic reconstruction, and divergence time estimation
187 (see below).

188

189 Phylogenetic reconstruction

190 We estimated relationships among haplotypes using maximum likelihood (ML) and Bayesian methods,
191 implemented in PHYLIP 3.69 (Felsenstein 1993) and Mr. Bayes 3.1.2 (Huelsenbeck and Ronquist 2001;

192 Ronquist and Huelsenbeck 2003) respectively. For computational efficiency, these analyses were carried
193 out on a subset of 24 unique haplotypes that included at least two samples from each lineage at each site
194 (two contact zone sites plus two reference sites for both *P. sherando* and *P. cinereus*).
195 We first estimated relationships among concatenated cyt-b and CO1 haplotypes. We used
196 genbank sequences for *Plethodon petraeus* (accession #s AY728222.1, NC_006334.1) as an outgroup.
197 Because CO1 sequences are not available for most *Plethodon*, we also estimated relationships among cyt-
198 b haplotpyes for an expanded set of species, including *P. shenandoah* (the Shenandoah salamander;
199 AY378043.1, AY378044.1, AY378045.1, AY378046.1), and *P. serratus* (the Southern Red-backed
200 salamander; DQ994981.1, DQ994982.1).
201 For maximum likelihood analyses, we used four gamma rate categories and a search strategy that
202 included iterations of branch length in all topologies. Five hundred bootstrap replicates were used to test
203 the support for each node. For the Bayesian analyses, we used 1,000,000 MCMC generations with
204 samples taken every 100 time steps. The first 25% of the parameter estimates and trees were discarded as
205 burn-in. To check for convergence, we ran four simultaneous chains, and in each case, chains converged
206 by 250,000 generations.

207 Divergence time

208 Time of divergence between *P. sherando* and *P. cinereus* was estimated using a coalescent-based
209 approach in BEAST 1.5.4. (Drummond and Rambaut 2007). We performed this analysis separately for
210 the two mitochondrial genes given their differing rates of evolution in plethodontid salamanders (Mueller
211 2006). We used a strict molecular clock model (Tajima's relative rates test, p=0.19). We took mean and
212 standard deviation rates of evolution from the fossil-calibrated plethodontid phylogeny of Mueller (2006):
213 1.04 (±0.27) substitutions per 100 million years for CO1 and 0.62 (±0.16) substitutions per 100 million
214 years for cytB. MCMC parameters were set to a chain length of 20 million with a burn-in of 2 million
215 and parameters logged every 1000 trees. Alteration of initial parameter values did not change the

resulting distribution for divergence time, suggesting that MCMC was run for a sufficient number of time steps.

Morphological analysis

We conducted several analyses of morphology to examine the distribution of phenotypes across contact zones and reference sites. First, we used linear discriminant function analysis (LDFA) 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 3 predictor variables - intercostal spaces per unit SVL, head width per unit SVL, and the presence or absence of brassy flecking (coded as 1/0). 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 that had been sequenced and assigned to a mitochondrial lineage (*P. sherando* or *P. cinereus*). Samples were assigned to two groups based on the morphological variables given above. These 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.

Results

P. sherando samples exhibited very low genetic diversity as compared to *P. cinereus* samples (Table 2). Across sites with no ambiguous bases, we recovered just 3 haplotypes with 3 variable nucleotide sites for *P. sherando*. Diversity was correspondingly low ($h = 0.41$, $\pi = 0.0009$, $k = 0.42$). *P. sherando* samples from just the two contact zones yielded similar estimates of diversity ($h = 0.56$, $\pi = 0.0010$, $k = 0.58$). In contrast, *P. cinereus* showed an order of magnitude greater nucleotide diversity within the same two contact zones (Table 2). We found 5 haplotypes with 14 variable sites ($h = 0.633$, $\pi = 0.0096$, $k = 5.52$). The comparatively low genetic diversity in *P. sherando* may not be typical of all mountaintop salamanders; *P. hubrichti* sequence diversity (6 samples from a single site) was also higher than that of *P. sherando* ($h = 0.60$, $\pi = 0.0051$, $k = 2.41$)

Genetic distance

P. 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 *P. sherando* and *P. cinereus* haplotypes was

strongly supported (100% bootstrap support and 100% posterior probability for each clade). *P. cinereus* samples grouped into two clusters that corresponded with the high elevation sites (PC2 and CZ2) and a cluster 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 Southern red-backed salamander (*Plethodon 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*).

Divergence time.

P. sherando and *P. cinereus* diverged about 12.7 million years ago based on Bayesian estimation for cyt-b (95% HPD 10.3-15.3). Estimated divergence time between these two species based on COI was somewhat lower (10.2 million years, 95% HPD 8.3 - 12.3). Divergence time between *P. sherando* and *P. serratus* was estimated as 6.0 million years (95% HDP 4.4-7.6). Given the position of *P. sherando* on the phylogeny reported above (i.e. sister to *P. serratus*), all of these estimates are consistent with the fossil-calibrated phlogeny of Wiens et al. (2006).

Morphological analysis

287 Morphology at reference sites was highly distinct between *P. sherando* and *P. cinereus*. Leave-one-out
288 cross validation at reference sites yielded 100% classification success by species/site. Contact zone
289 samples were, for the most part, similarly distinct. When individuals were classified based on
290 morphology and validated based on mtDNA lineage, classification success was 98% for each lineage (Fig
291 4). One *P. sherando* genotype was classified as *P. cinereus* and one *P. cinereus* genotype was classified
292 as *P. sherando*. These 2 individuals were both juveniles, and lower posterior probabilities for other
293 juveniles in the sample suggested that the morphological model may have been less reliable for juveniles.
294 ANOVA indicated that, overall, salamanders in the contact zone were at least as distinct as salamanders at
295 the reference sites. Interactions between lineage (*P. sherando* or *P. cinereus*) and site type (reference site
296 or contact zone) were non-significant for both intercostal spaces/SVL ($F_{1,109} = 0.69$, $p = 0.41$) and for
297 head width/SVL ($F = 2.97$, $p = 0.08$). We note that the marginal result for head width ($p = 0.08$) is in the
298 opposite direction than would be predicted with hybridization - lineages in the contact zone were more
299 different than were lineages at the reference sites (Fig 5). This result is plausibly consistent with findings
300 of character displacement in head shape at contact zones between other pairs *Plethodon* (e.g. Adams
301 2004, Adams et al. 2007). Follow-up analysis showed that this pattern for head shape was in fact
302 statistically significant ($F_{1,219} = 4.74$, $p = 0.03$) when we increased our sample size by classifying
303 salamanders by the number of intercostal spaces (for which we had many more measurements) instead of
304 their mtDNA lineage.
305

306 **Discussion**

307

308 Prior work on *Plethodon sherando* (Highton 2004) suggested it to be a distinct lineage based on
309 morphological and allozymic differentiation from *P. cinereus*. In the current study, we show that *P.*
310 *sherando* is highly distinct from *P. cinereus* in their mtDNA sequences, with 17% sequence divergence

311 across two loci. Our analysis of morphology in contact zones and reference sites further supported the
312 distinction between these two taxa. All but two individuals were correctly assigned as *P. sherando* or *P.*
313 *cinereus* on the basis of a few morphological characters, and no overall evidence of morphological
314 intermediates was detected in contact zones. The concordance between mitochondrial genotype and
315 morphology suggests that there has been little recent introgression between the lineages where they live in
316 sympatry. Our phylogenetic analyses further supported this conclusion; *P. sherando* and *P. cinereus*
317 lineages were reciprocally monophyletic using both maximum likelihood and Bayesian methods. These
318 findings, combined with the very low genetic diversity found in *P. sherando*, suggest that *P. sherando* has
319 a distinct evolutionary history and has probably undergone some period of isolation on or near the Big
320 Levels ridgetop.
321 *Plethodon sherando's* extremely limited distribution (<80 km^2) and its rapid replacement by *P.*
322 *cinereus* along its range boundaries create several potential conservation concerns for this species. In
323 general, anytime a species with a restricted distribution is surrounded by a more common congener,
324 genetic swamping via introgression may be considered as a potential threat (Chan et al. 2006; Rhymer
325 and Simberloff 1996). Moreover, many other *Plethodon* species are known to hybridize (Highton 1998;
326 Highton 1999) and hybridization may lead to substantial introgression in these species (Wiens et al. 2006;
327 Weisrock et al. 2005). With *P. sherando*, we find little evidence for hybridization at contact zones and
328 we suggest that the threat of "genetic swamping" by *P. cinereus* does not appear to be a pressing concern.
329 Further studies at other contact zones between these two species would be helpful to ensure that
330 introgression is not occurring extensively at other sites within *P. sherando*'s restricted range.
331 One of the most critical threats to mountaintop endemics like *P. sherando* may be climate change
332 coupled with range expansion by competitors (Gilman et al. 2010). Milanovich et al. (2010) modeled
333 salamander persistence in the Southern Appalachians as a function of climate variables and showed that
334 climate warming could lead to major range contractions. Their study did not include salamanders of the
335 *P. cinereus* group; however, this group is also known to be sensitive to climate. Arif et al. (2007) argued,

based on ecological niche models, 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 simple 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 this range includes both high elevations within the Blue Ridge and colder regions as far north as central Quebec (Petranka 1998). Thus, more research is necessary to determine whether warmer conditions would likely favor *P. cinereus* over *P. sherando*.

Our study was not designed to examine population history in that we did not sample across the range of *P. sherando*, and our *P. sherando* sequences did not contain enough genetic diversity to calculate common metrics for population expansion or contraction (e.g. mismatch distributions). We can, however, examine the genetic structure of *P. cinereus* in contact zones versus reference sites and ask whether these analyses suggest that contact zones might represent areas where red-backed salamanders 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 haplotypes given the level of nucleotide diversity (negative values for Fu's F_s), and a unimodal mismatch distribution (Harpending 1994; Johnson et al. 2007; Schneider and Excoffier 1999). Examining our data, we found that the lower contact zone site consisted of only a single *P. cinereus* haplotype, whereas the mean number of pairwise differences (π) in the upper contact zone was 0.33, as compared to $\pi = 0.13$ and 0.78 in the two *P. cinereus* reference sites. Fu's F_s could not be calculated for the lower contact zone (since there were no rare haplotypes), but F_s was significantly negative in the upper contact zone ($F_s = -1.32$, $p = 0.007$) whereas it was not significantly different from zero for either of the reference sites ($F_s = -0.65$, $p = 0.11$, and $F_s = 0.41$, $p = 0.55$). Harpending's raggedness index (which indicates departure from a unimodal distribution) was less informative - it only

approached significance for one of the reference sites ($p = 0.07$), indicating that an expansion model could not be rejected at any of the sites. Collectively, these results do provide some evidence that red-backed salamanders might already be expanding into the range of *P. sherando*. That said, these analyses are quite preliminary, as sample sizes for each of these sites are small ($n = 12$-15) and the power of these tests is low. Additionally, dispersal by red-backs into new habitats may be a very slow process (Cabe et al. 2007) such that these indices of population expansion may not be sufficiently sensitive to detect recent shifts in the distribution of *P. cinereus*.

The phylogentic analysis of *P. sherando* is interesting in that it suggests that, unlike *Plethodon 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 Southern Red-backed salamander, *P. serratus* (Fig 3). Although statistical support for this node was only moderate (79% ML boostrap 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). The Southern Redback's current range includes parts of Arkansas, Missouri, North Carolina Alabama, Georgia, Louisiana and Oklahoma (Petranka 1998). At the Northeastern 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 more Northern *P. serratus* ancestor shifted southwards.

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

386 morphological evidence for hybridization at these sites, it is possible that hybridization was occurring at
387 other sites where we did not collect. For example, morphological and allozymic data suggest
388 hybridization between *P. shenandoah* and *P. cinereus* at one site but not at two others (Highton 1967;
389 Highton 1999). A third limitation is that we sampled only a portion of *P. sherando's* range. Although we
390 sampled opposite ends of the range, it is possible that we missed pockets of genetic diversity that would
391 have changed our understanding of the nature and history of the *P. sherando* lineage. Finally, with only
392 two mitochondrial loci, it is possible that gene trees could differ substantially from true species trees
393 (Degnan and Rosenberg 2009). That said, our results agree with the results of prior studies based on
394 allozymes (Highton 2004) and forthcoming phylogenies of the *Plethodon* genus based on a large number
395 of nuclear and mitochondrial markers (R. Highton, personal communication).
396 In spite of these limitations, our study has clear conservation implications. *Plethodon sherando*
397 currently lacks any special status within the U.S. or the State of Virginia because of its very recent
398 recognition and a lack of knowledge of its biology. In contrast, the other three mountaintop salamanders
399 in the region all have some conservation status. The Shenandoah salamander (*P. Shenandoah*) is listed as
400 Federally Endangered and thus protected by the U.S. Endangered Species Act. The Cheat Mountain
401 salamander (*P. nettingi*) is Federally Threatened and covered by a formal recovery plan. The Peaks of
402 Otter salamander (*Plethodon hubrichti*) is listed federally as a "species at risk," and is state-listed as a
403 "species of concern." This special status for *P. hubrichti* requires the consideration of management
404 impacts on the species where it occurs within public lands. For example, forestry within the range of *P.*
405 *hubricti* is scheduled outside the normal surface activity periods for the species, and shelterwood and
406 course woody debris is retained at sites where forestry occurs. Based on our findings, we suggest that
407 *Plethodon sherando* should merit a similar status to that of *P. hubrichti*. The range sizes of the two
408 species are similar (~100 km^2 for *P. hubrichti* versus ~80 km^2 for *P. sherando*), and both species have
409 ranges that consist primarily of National Forest land. Moreover, both species' distributions are entirely
410 surrounded by red-backed salamanders, suggesting a similar set of conservation concerns with respect to

411 competition and climate change. Our results show that *P. sherando* is at least as distinct genetically from
412 other salamanders in the region as is *P. hubrichti* and that *P. sherando* is substantially more distinct from
413 *P. cinereus* than is *P. shenandoah*. Although a number of different criteria for Evolutionary Significant
414 Units (ESUs) have been proposed (Fraser and Bernatchez 2001; Goldstein et al. 2000), *Plethodon*
415 *sherando* would appear to represent an ESU under any of these criteria.

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Table 1. Genetic diversity for different species and sets of sampling locations. "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 "k" is the mean number of differences between pairs of sequences.

Species	Sites	n	haplotypes	var sites	h	π	k
P. sherando	All	62	3	3	0.41	0.0009	0.42
P. sherando	Contact zones	31	3	3	0.56	0.0010	0.58
P. cinereus	All	55	6	16	0.65	0.0082	4.47
P. cinereus	Contact zones	24	5	14	0.63	0.0096	5.52
P. hubrichti	All*	5	4	10	0.90	0.0089	5.20

* Five samples from Peaks of Otter salamanders at two sites near the Eastern end of their range

Figure Legends

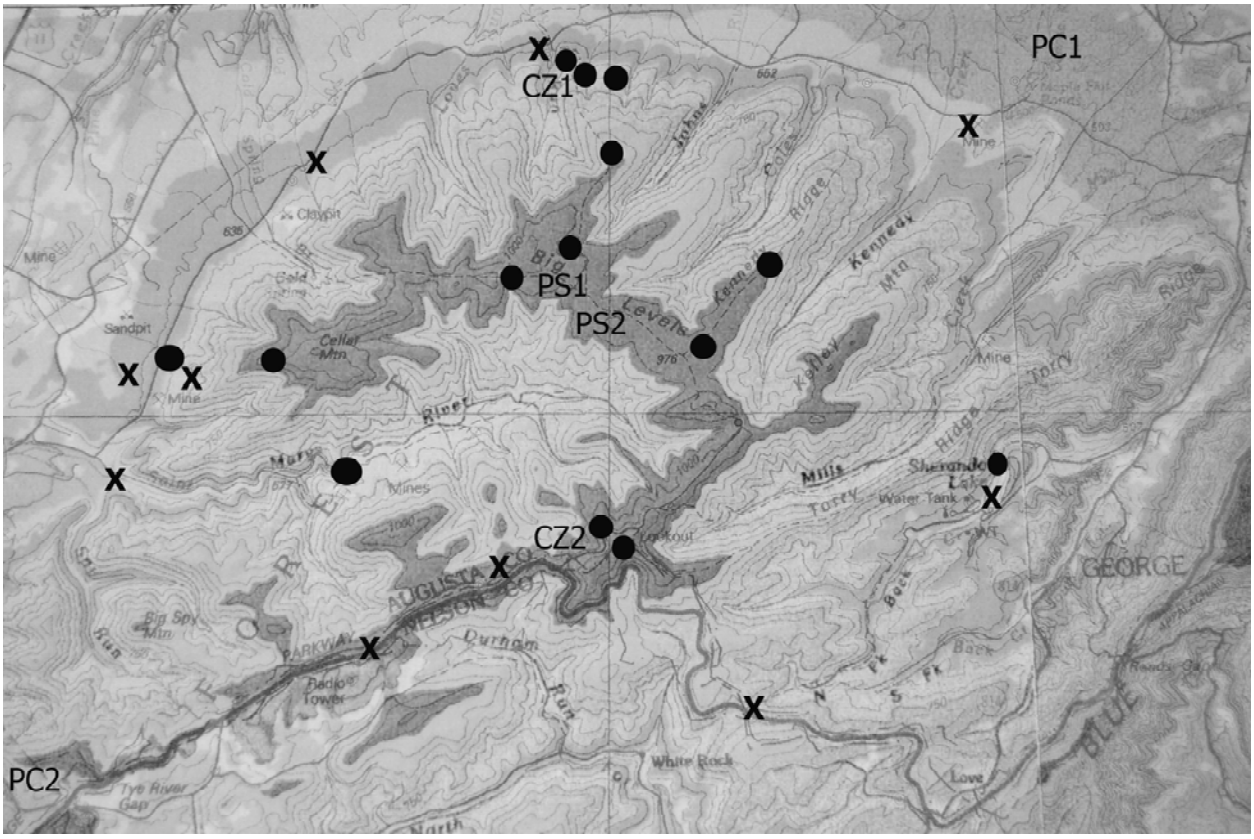
Fig 1 Map of the range of *Plethodon sherando* and our sampling sites. Filled circles indicate sites where *P. sherando* was found by Highton (2004) or in our own surveys. "X"s indicate sites where *P. cinereus* but not *P. sherando* was found. PS1 and PS2 indicate *P. sherando* reference sites one and two, PC1 and PC2 indicate *P. cinereus* reference sites, and CZ1 and CZ2 are two contact zone sites where both species were found. Shading corresponds to elevation, with the darkened area indicating the Big Levels ridge

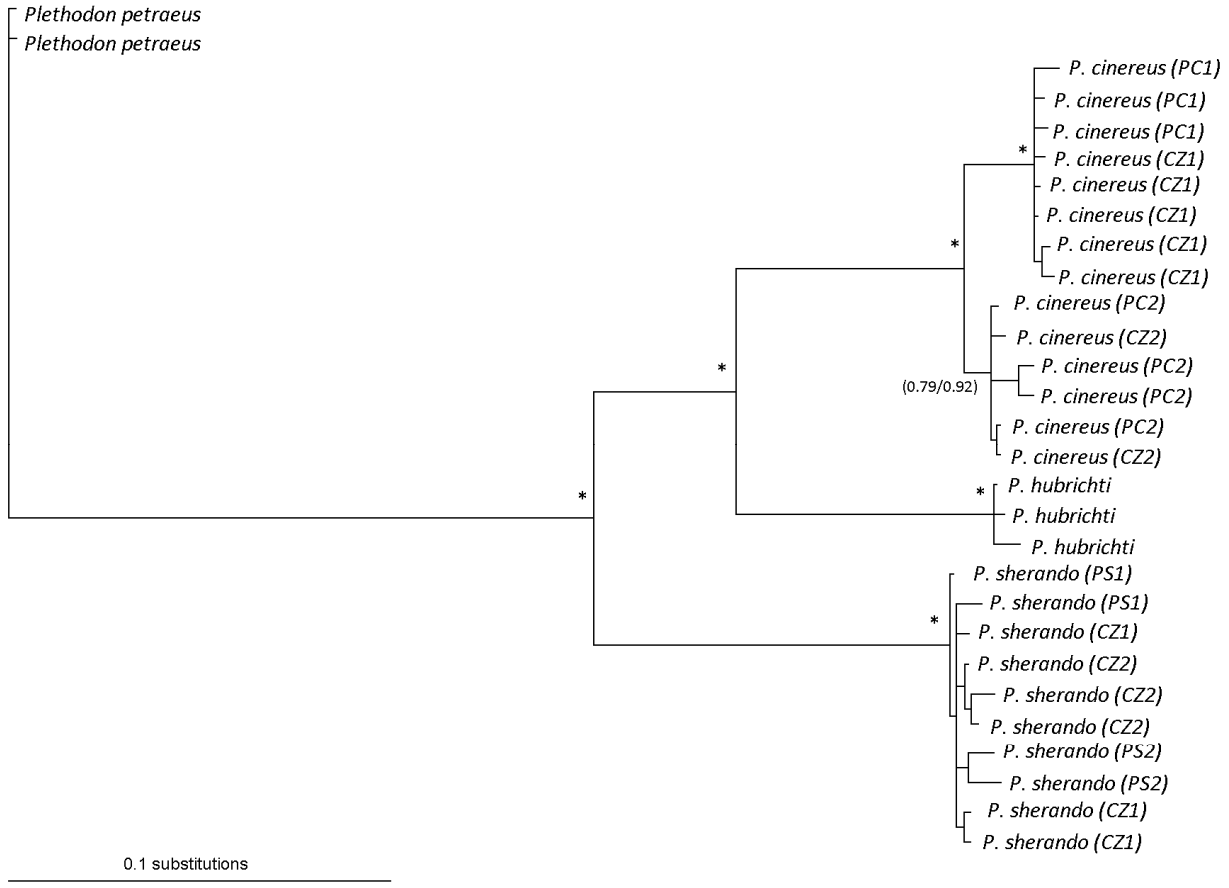
Fig 2 Maximum likelihood and Bayesian phylogeny based on concatenated cyt-b and CO1 sequences with *Plethodon petraeus* used as an outgroup. Numbers in parentheses are bootstrap support (proportion of bootstrap replicates) and posterior probabilities for each node. Nodes with support > 0.98 are indicated with an "*". Site locations for samples (see Figure 1) are given for each sample

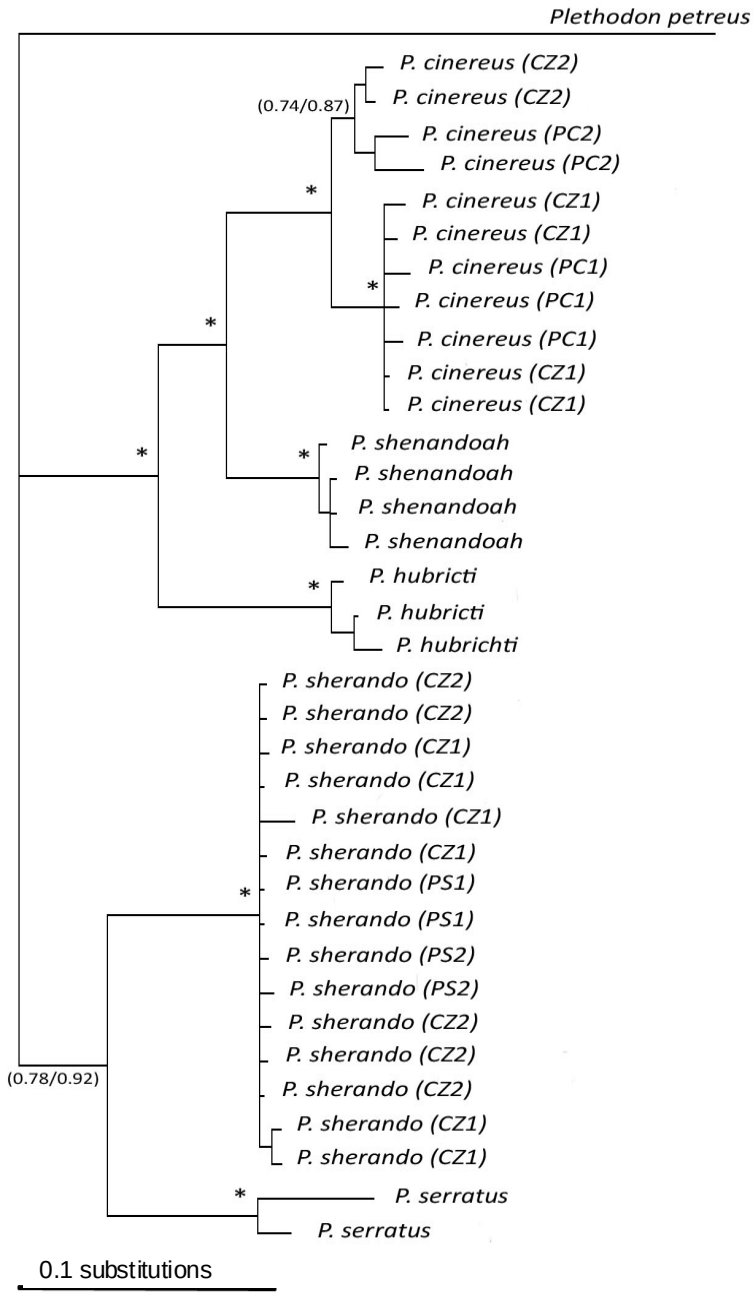
Fig 3 Maximum likelihood and Bayesian phylogeny for cyt-b with *Plethodon petraeus* used as an outgroup. Numbers in parentheses are bootstrap support (proportion of bootstrap replicates) and posterior probabilities for each node. Nodes with support > 0.98 are indicated with an "*". Site locations for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

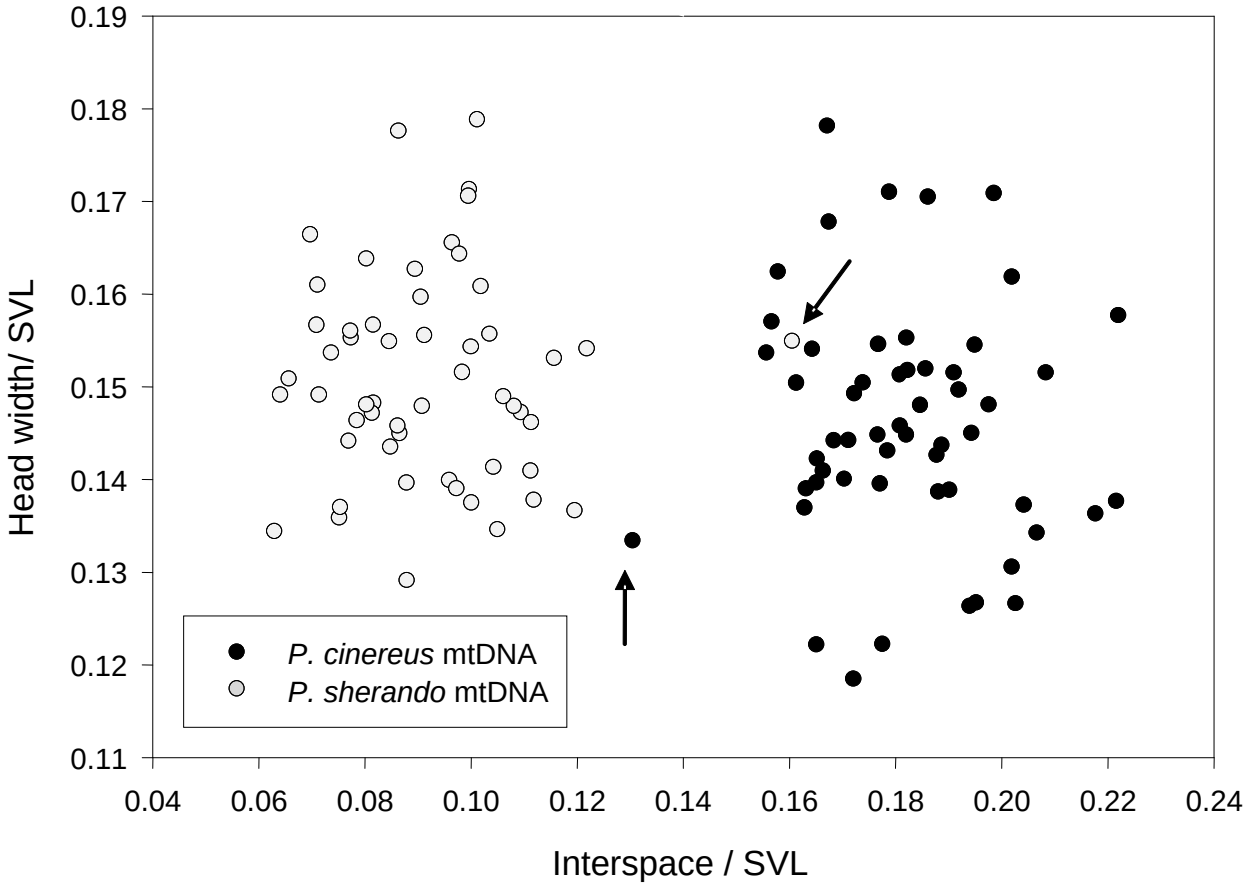
Fig 4 Results for classification of contact zone salamanders with known mtDNA lineages based on morphological characters. All morphological classifications were consistent with mtDNA lineages except for the two indicated with arrows

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

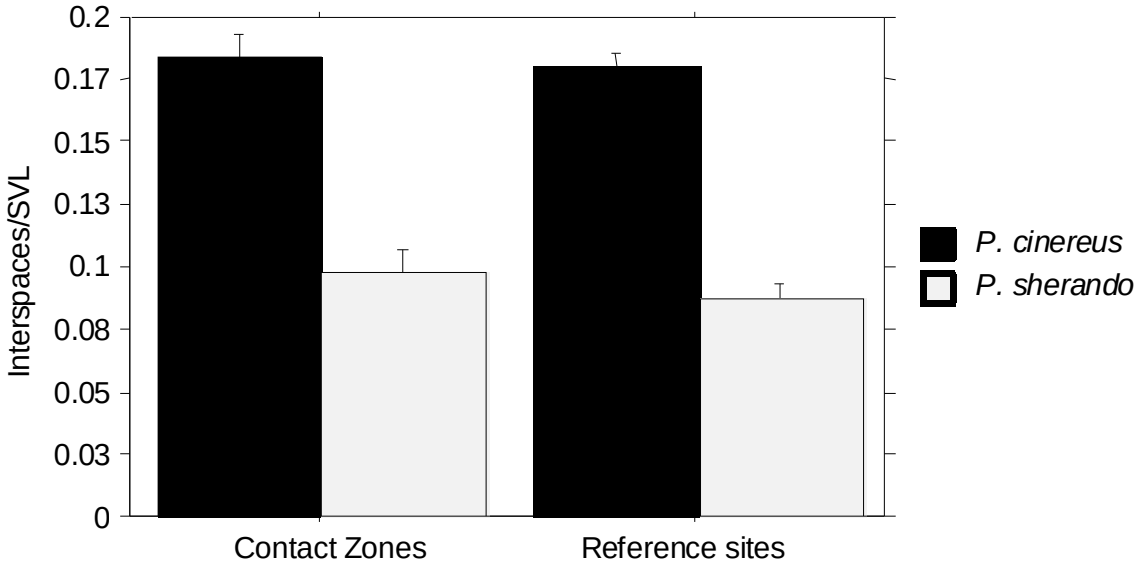

PC1
CZ1
PS1
PS2
CZ2
PC2
2 km


Plethodon petraeus
Plethodon petraeus
P. cinereus (PC1)
P. cinereus (PC1)
P. cinereus (PC1)
P. cinereus (CZ1)
P. cinereus (CZ1)
P. cinereus (CZ1)
P. cinereus (CZ1)
P. cinereus (CZ1)
P. cinereus (PC2)
P. cinereus (CZ2)
P. cinereus (PC2)
P. cinereus (PC2)
(0.79/0.92)
P. cinereus (PC2)
P. cinereus (CZ2)
P. hubrichti
P. hubrichti
P. hubrichti
P. sherando (PS1)
P. sherando (PS1)
P. sherando (CZ1)
P. sherando (CZ2)
P. sherando (CZ2)
P. sherando (CZ2)
P. sherando (PS2)
P. sherando (PS2)
P. sherando (CZ1)
P. sherando (CZ1)
0.1 substitutions


Plethodon petreus
P. cinereus (CZ2)
P. cinereus (CZ2)
(0.74/0.87)
P. cinereus (PC2)
P. cinereus (PC2)
P. cinereus (CZ1)
P. cinereus (CZ1)
P. cinereus (PC1)
P. cinereus (PC1)
P. cinereus (PC1)
P. cinereus (CZ1)
P. cinereus (CZ1)
P. shenandoah
P. shenandoah
P. shenandoah
P. shenandoah
P. hubricti
P. hubricti
P. hubrichti
P. sherando (CZ2)
P. sherando (CZ2)
P. sherando (CZ1)
P. sherando (CZ1)
P. sherando (CZ1)
P. sherando (CZ1)
P. sherando (PS1)
P. sherando (PS1)
P. sherando (PS2)
P. sherando (PS2)
P. sherando (CZ2)
P. sherando (CZ2)
P. sherando (CZ2)
(0.78/0.92)
P. sherando (CZ1)
P. sherando (CZ1)
P. serratus
P. serratus
0.1 substitutions

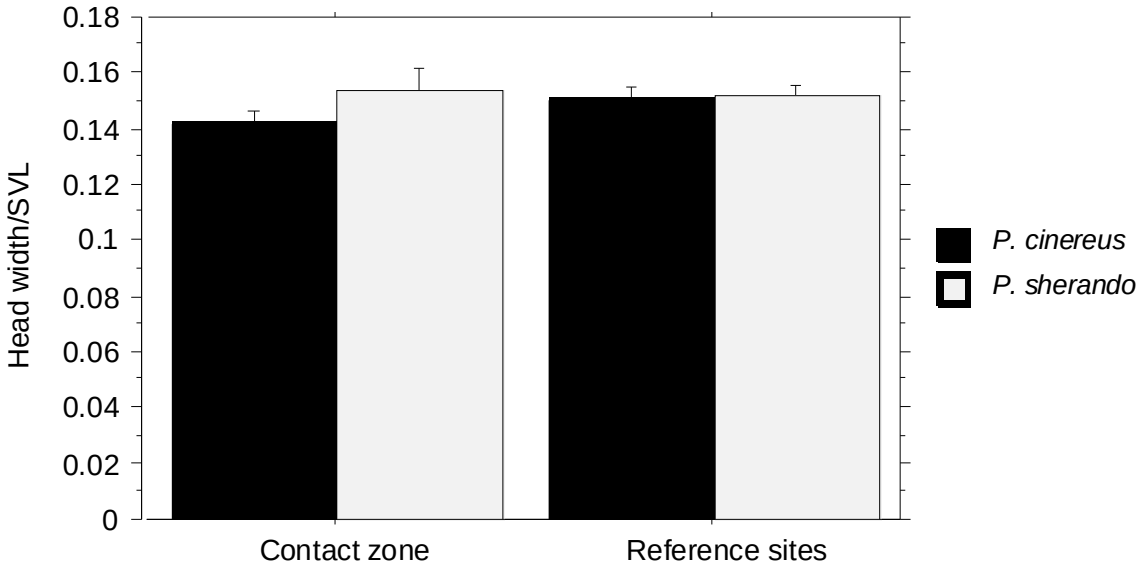

0.19
0.18
0.17
0.16
0.15
0.14
0.13
0.12
0.11
Head width/ SVL
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0.22
0.24
Interspace / SVL
P. cinereus mtDNA
P. sherando mtDNA

A



Site by species interaction: p = 0.41

B.



Site by species interaction, p = 0.08