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Conservation genetics of the endangered Shenandoah salamander (*Plethodon shenandoah*, Plethodontidae)

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Abstract

The Shenandoah salamander (*Plethodon shenandoah*) is restricted to three isolated talus outcrops in Shenandoah National Park, VA, USA and has one of the smallest ranges of any tetrapod vertebrate. This species was listed as endangered under the US Endangered Species Act in 1989 over concern that direct competition with the red-backed salamander (*Plethodon cinereus*), successional habitat changes, and human impacts may cause its decline and possible extinction. We address two issues herein: (1) whether extensive introgression (through long-term hybridization) is present between the two species and threatens the survival of *P. shenandoah*, and (2) the level of population structure within *P. shenandoah.* We provide evidence from mtDNA haplotypes that shows no genetic differentiation among the three isolates of *P. shenandoah*, suggesting that their fragmentation is a geologically recent event, and/or that the isolates are still connected by occasional gene flow. There is also no evidence for extensive introgression of alleles in either direction between *P. cinereus* and *P. shenandoah*, which suggests that *P. shenandoah* may not be in danger of being genetically swamped out through hybridization with *P. cinereus*.

INTRODUCTION

Plethodon shenandoah (Amphibia: Plethodontidae) was described on the basis of morphological criteria (Highton & Worthington, 1967), and was considered to be a member of the *P. cinereus* group. Compared to *P. cinereus*, *P. shenandoah* has a lower number of trunk vertebrae (19 vs. 20), a darker venter, a striped phase with a narrower dorsal stripe, and an unstriped phase with reduced brassy iridophore flecking and scattered red pigment in the mid-dorsal region (Highton & Worthington, 1967). Highton (1988) reported that *P. shenandoah* was confined to northwest-facing talus outcrops occupying less than 6 km^2 on three of the highest mountain peaks in Shenandoah National Park, VA, USA. It thus has one of the smallest ranges of any tetrapod vertebrate. Recently, new populations were reported further to the southwest (Thurow, 1999), though their identity remains to be confirmed genetically. Recent protein electrophoretic studies confirm the genetic distinctiveness of the three populations of *P. shenandoah* relative to *P. cinereus* in Shenandoah National Park (Highton, 1999), but no study has yet employed any other class of molecular marker.

At two of the localities, *P. shenandoah* is surrounded by *P. cinereus*, which occupies the more mesic leaf litter communities that border the talus outcrops (Highton, 1988). These two species have become well known among ecologists as a model system for studies of competitive exclusion. *Plethodon cinereus* was found to be the better competitor in moist deep soils, restricting *P. shenandoah* to the dry talus outcrops where it has a physiological advantage (Jaeger, 1970, 1971*a*,*b*, 1972, 1980; Wrobel, Gergits & Jaeger, 1980; Bobka, Jaeger & McNaught, 1981; Gergits & Jaeger, 1990). Highton & Worthington (1967) indicated that some individuals of *P. cinereus* resembled *P. shenandoah* at a point of parapatric contact on the northeastern edge of the Hawksbill Mountain isolate, and interpreted these observations as morphological evidence for hybridization at this locality.

Because of its limited distribution, *P. shenandoah* was listed as state endangered by the Commonwealth of Virginia in 1987 (Wynn, 1991) and federally endangered under the US Endangered Species Act in 1989 (54 FR34464, US Fish and Wildlife Service [USFWS], 1994). The species was initially thought to be endangered by natural processes such as direct competition with *P. cinereus* (Jaeger, 1970), and indirectly by successional changes (weathering and soil formation) within the *P. shenandoah* isolates, which would eventually make conditions more favorable for *P. cinereus*. Other factors which may threaten the isolates of *P. shenandoah* include: (1) forest defoliation (due to the intro-

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duced gypsy moth (*Lymantria dispar*) and hemlock woolly adelgid (*Adelges tsugae*)); (2) impacts associated with acid deposition and/or precipitation; (3) routine activities carried out by the National Park Service, such as maintenance of roads and trails, and fire management. The Shenandoah Salamander Recovery Plan (USFWS, 1994) outlined a number of tasks aimed at further identifying and mitigating these threats. Three of these recovery tasks can be addressed by analyzing molecular genetic data from populations of *P. shenandoah* and *P. cinereus* within Shenandoah National Park (task 1.0 ('Continue searches of appropriate habitat to define boundaries of existing populations and to determine whether additional populations exist'; p. 13), task 3.2 ('Evaluation of effects of roads and trails on movements on Shenandoah salamanders and potential population fragmentation'; p. 16), and task 4.3 ('Continue to support studies defining the interactions of *P. cinereus* and *P. shenandoah*'; p. 21, USFWS, 1994)).

We addressed the tasks above by sampling mtDNA haplotypes (alleles) and estimating genetic variability within the three populations of *P. shenandoah* relative to nearby populations of *P. cinereus*. With respect to tasks 1.0 and 3.2, we first asked whether the existing three isolates of *P. shenandoah* show any evidence of long-term historical fragmentation. If we had found that the mtDNA haplotypes were represented by three monophyletic groups that correlate strongly with geography (i.e., monophyly of haplotypes within each of the three populations), then we would infer that these populations had undergone fragmentation in the distant past. That is, they comprise three distinct and evolutionarily independent populations (Moritz, 1994), and their present disjunct distribution may not, in and of itself, pose any threat to their persistence. If, however, non-monophyly of haplotypes among groups is evident (i.e., the pattern shows a mixture of haplotypes among all three populations), the implication is that their current isolation is of relatively recent origin. This result would be consistent with patterns of allozyme variation recently described by Highton (1999). In this case, if population sizes in any of the isolates are small and have not yet attained a new equilibrium (between mutation, drift, and extinction of alleles), then they may be vulnerable to extinction if human activity increases their susceptibility to genetic, demographic or stochastic processes (Lande, 1988). Ongoing gene flow might also explain the mixture of haplotypes among the three populations of *P. shenandoah*, which would make them less vulnerable to extinction. However, ongoing gene flow among the three populations may not be extensive given that *P. shenandoah* appears completely restricted to the talus habitat due to competitive exclusion by *P. cinereus* (Jaeger, 1970, 1971*a*, *b*, 1972, 1980; Wrobel *et al*., 1980; Bobka *et al*., 1981; Highton, 1988, 1999; Griffis & Jaeger, 1998).

The issue of interactions and hybridization between *P. shenandoah* and *P. cinereus* (task 4.3 in the Recovery Plan) can be addressed by determining whether or not alleles present in populations of one species have introgressed into populations of the other, and to what degree and in which direction introgression may be occurring. Available data lead to different expectations among the three isolates. For example, allozyme data (unpublished; cited in Highton, 1999) corroborate the morphological evidence for hybridization at the edge of the Hawksbill Mountain site (Highton & Worthington, 1967). These data suggest that if hybridization leads to ongoing introgression, or is a cause of past introgression between these species, an appropriate sampling design (local transects through points of contact) should reveal the genetic consequences of this interaction. Although hybrids have not been reported at either Stony Man Mountain or The Pinnacles, samples of *P. shenandoah* at Stony Man also share alleles (allozymes) with *P. cinereus*, and the high heterozygosities at both sites are consistent with interpretations of ongoing (Hawksbill) or historical (Stony Man) introgression (Highton, 1999). At the third locality (Pinnacles), the two species are syntopic throughout the isolate and there is no morphological or allozyme evidence for hybridization (Highton, 1999). If hybridization between *P. cinereus* and *P. shenandoah* at the Hawksbill or Stony Man locations has been historically or currently sufficient for introgression to occur, we might expect the gene pool of *P. shenandoah* at these sites to be introgressed with alleles from the surrounding *P. cinereus*. Alternatively, syntopy between the two species at the Pinnacles site, in the absence of any evidence for hybridization, should be manifested as a 'non-introgressed' *P. shenandoah* gene pool.

MATERIALS AND METHODS

Tissue samples from ten individuals of *P. shenandoah* were collected from the interior of each of the three populations at Pinnacles (5–24–99), Stony Man (5–4–99), and Hawksbill Mountain (5–5–99) in Shenandoah National Park (Fig. 1), well away from known or potential hybrid zones at range edges. Equivalent samples were also taken from ten individuals of *P. cinereus* collected 150–490 m away from the collection sites of *P. shenandoah* at the Stony Man Mountain (10–8–99), Hawksbill Mountain (10–8–99) and Pinnacles sites (10–9–99) (Fig. 1). Four specimens of *Plethodon hubrichti* were collected in Jefferson National Forest (10–19–99, Bedford County, VA) for use as an outgroup not only to polarize the characters, but also to statistically test for reciprocal monophyly of mtDNA alleles between *P. cinereus* and *P. shenandoah*. Salamanders were captured by hand and photographed, and approximately 1 cm (or less) of tail tip was clipped from each individual and preserved in Nunc tubes with 95% ethanol. Salamanders were then released at their point of capture. Several individuals were omitted from the analysis owing to our inability to get clean sequence from the PCR products. Total numbers of individuals of each species at each locality are presented in Table 1.

Genomic DNA was extracted from each tail tip using a protocol described by Crandall *et al*. (1999*a*). This consisted of using a cell lysis solution and proteinase-K to

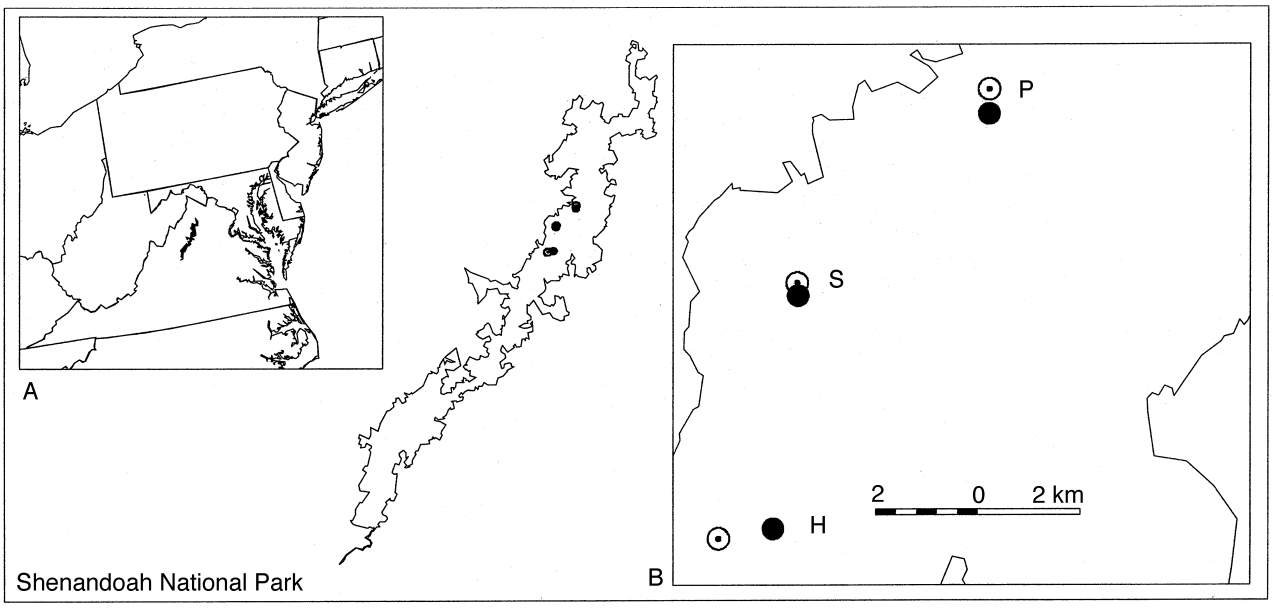

Fig. 1. Location of Shenandoah National Park in the Blue Ridge region of Virginia (solid area in left panel), and the location of the three isolated populations of *P. shenandoah* in the park (symbols on park map in the middle panel). The right panel shows the approximate collecting areas for *P. cinereus* (open circles) and *P. shenandoah* (closed circles) at the Pinnacles (P), Stony Man Mountain (S) and Hawksbill Mountain (H) locations.

digest the tissue, followed by protein precipitation with ammonium acetate buffer, and then DNA precipitation in isopropanol. DNA was resuspended in 50–100 μl of TE buffer (depending on the amount of DNA recovered during the extraction) and stored at 4°C until use.

A fragment of 404 bp of the mitochondrial cytochrome-*b* gene was amplified for all individuals using MVZ15 (Moritz, Schneider & Wake, 1992) and cyt-*b*2 (Kocher *et al*., 1989) primers. Amplifications by PCR were carried out in 25 μl reactions: 2.5 μl 10X reaction buffer, 1.5 μl MgCl, 4 μl dNTP, 0.15 μl *Taq* polymerase, 13.35 μl H_20, and 1 μl genomic DNA as template. All amplifications were carried out in a Perkin Elmer 9600 thermal cycler. The PCR amplification protocol began with an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. A final extension followed at 72°C for 5 minutes. PCR products were then purified using a GeneClean II Kit (Bio 101, Inc., Vista, CA).

DNA sequences were generated on an ABI 377XL automated sequencer using the ABI Big-dye Ready-Reaction kit and following their standard cycle sequencing protocol (PE Applied Biosystems, Foster City, CA). Sequences were aligned using Sequencher 3.1.1 (Gene Codes Corp. Inc., 1995) and imported into PAUP*4.0b4a (Swofford, 1998) for phylogenetic analysis. Sequences are available in GenBank under accession numbers AF302845–AF302902.

Regardless of the criteria being used for phylogenetic reconstruction, one explicitly or implicitly assumes a model of evolution. As increasingly complex models of evolution are being developed for DNA sequence data, it can be an advantage to use these more parameter rich models (Goldman, 1993*a*; Goldman & Yang, 1994; Yang, Goldman & Friday, 1994). However, determining how complex a model is needed given one's data is a problem of statistical inference (Goldman, 1993*b*; Rzhetsky & Nei, 1995; Huelsenbeck & Crandall, 1997). In order to determine the appropriate model of evolution for the cytochrome *b* sequences used in this analysis, the computer program MODELTEST 3.01 (Posada & Crandall, 1998) was used to test a number of null hypotheses about the sequence data, including whether: (1) nucleotide frequencies are equal (or not), (2) mutation rates for transitions are equal (or not) to those for transversions, (3) rates are equal (or not) within transitions and transversions, (4) rates are homogeneous across all variable sites within the data set, and (5) the sequences contain a significant proportion of invariable sites. MODELTEST evaluates paired alternative models in a hierarchical framework (from simple to more parameter rich models) to optimize the fit of data to a model, given an initial neighbor-joining (NJ) tree with the assumptions of the Jukes–Cantor (1969) model.

Table 2 summarizes these paired tests, and for *Plethodon* cytochrome *b* sequences the HKY85 model (Hasegawa, Kishino & Yano, 1985) was selected as the optimal fit given the data. In other words, the mutational dynamics of these sequences are adequately explained by a model of evolution based on assumptions of unequal base frequencies, unequal Ti/Tv rates, equal rates within transitions and transversions, equal rates among sites, and no invariable sites. More complex models offer no significant improvement in explanatory power (Table 2).

Using PAUP*4.0b4a (Swofford, 1998), maximum likelihood (ML) and maximum parsimony (MP)

Table 1. Distribution of mtDNA haplotypes and protein electromorphs (lower case letters with frequencies in parentheses; summarized from Appendix II in Highton, 1999) for all *Plethodon* populations sampled in Shenandoah National Park (H = Hawksbill, P = Pinnacles, S = Stony Man). Expected heterozygosity values averaged across all allozyme data are included.

Taxa	*P. cinereus*			*P. shenandoah*		
Localities	H	S	P	H	S	P
mtDNA						
Sample sizes	8	9	9	10	9	9
P. cinereus (H1)	0.375	–	–			
P. cinereus (H2)	0.125	–	–			
P. cinereus (H7)	0.125	–	–			
P. cinereus (H8)	0.125	–	–			
P. cinereus (S8)	0.250	0.11	–			
P. cinereus (P9)	–	0.89	1.00			
P. shenandoah (H2)				0.10	–	–
P. shenandoah (H10)				0.90	0.78	1.00
P. shenandoah (S10)				–	0.22	–
Protein loci (Highton, 1999)						
Sample sizes	–	–	30	32	33	32
α-GPD	–	–	b(1.00)	b(0.64)	b(0.59)	b(0.73)
			c(0.36)	c(0.41)	c(0.27)	
Est-β	–	–	h(0.02)	f(0.08)	f(0.02)	
			n(0.98)	g(0.02)		g(0.03)
				j(0.28)	j(0.71)	j(0.71)
				m(0.02)		
				o(0.31)	o(0.07)	o(0.23)
				p(0.09)		
				q(0.03)	q(0.03)	q(0.01)
				r(0.03)		
				s(0.14)	s(0.17)	s(0.02)
PGDH	–	–	c(1.00)	c(0.88)	c(0.81)	c(1.00)
			a(0.12)	a(0.19)		
TRF	–	–	g(0.94)	d(0.73)	d(0.61)	d(0.32)
			J(0.06)	h(0.27)	h(0.31)	h(0.68)
					a(0.01)	
					b(0.02)	
					c(0.05)	
IDH-1	–	–	b(0.75)	b(0.84)	b(0.73)	b(0.83)
			d(0.25)	d(0.16)	d(0.27)	d(0.17)
IDH-2			b(0.05)	b(0.25)	b(0.33)	b(0.15)
			d(0.95)	d(0.75)	d(0.67)	d(0.85)
AAT-1	–	–	d(0.05)	d(0.97)	d(1.00)	d(1.00)
			e(0.65)	e(0.02)		
			b(0.30)	f(0.01)		
LDH-H	–	–	c(1.00)	c(1.00)	c(0.99)	c(1.00)
					a(0.01)	
PGM			b(1.00)	b(1.00)	b(1.00)	b(0.98)
						c(0.02)
Expected heterozygosity (h) averaged across all loci			0.123	0.284	0.302	0.205

Table 2. MODELTEST analysis of 56 hierarchical substitution models for the *Plethodon* mtDNA data. –lnL scores were estimated under various models of evolution on a neighbour-joining tree and compared for the best fit to the sequences as described by Posada & Crandall (1998).

Null model (H_0)	H_0 vs. H_1	$-\ln L_0$	$-\ln L_1$	df	*P*
equal base frequencies	JC69[a] vs. F81[b]	951.70	923.78	3	<0.000001
equal ti/tv rates	F81[b] vs. HKY85[c]	923.78	881.26	1	<0.000001
equal ti rates	HKY85[c] vs. TrN[d]	881.26	880.58	1	0.244856
equal tv rates	HKY85[c] vs. K81uf[e]	881.26	881.17	1	0.681596
equal rates among sites	HKY85[c] vs. HKY85 + Υ[f]	881.26	880.28	1	0.162711
no invariable sites	HKY85[c] vs. HKY85 + I[g]	881.26	880.23	1	0.152116

[a]JC69, Jukes & Cantor (1969)
[b]F81, Felsenstein (1981)
[c]HKY85, Hasegawa *et al.* (1985)
[d]TrN, Tamura & Nei (1993)
[e]K81uf, Kimura with unequal base frequencies (1981)
[f]Υ, shape parameter of the gamma distribution
[g]I, proportion of invariable sites

optimality criteria were used to reconstruct haplotype relationships. For the MP analysis, all characters were equally weighted and an exhaustive search was performed. We also performed a NJ analysis, based on genetic distances calculated using the estimates from the HKY85 model (Ti/Tv ratio = 5.3480, base frequencies A = .3487, C = .1252, G = .2262, T = .2999). The ML tree was reconstructed using the same parameters and using the branch-and-bound search algorithm. In all cases, *P. hubrichti* was used as the outgroup, and support for internal nodes was assessed by nonparametric bootstrap analysis (Felsenstein, 1985) with 1000 pseudoreplicates.

We used the computer programs COALESCE (Kuhner, Yamato & Felsenstein, 1995) and FLUCTUATE (Kuhner, Yamato & Felsenstein, 1998) to estimate genetic diversity ($\theta = 4N_e\mu$) within both species under assumptions of constant and fluctuating population sizes, respectively. By using the estimator θ (a product of inbreeding effective population size [N_{ei}] and the mutation rate), and comparing values of θ between *P. shenandoah* and *P. cinereus*, we avoid the issue of estimating N_{ei} directly if we assume an equal mutation rate for both species. For both programs, parameter estimates for Ti/Tv ratio and base frequencies were retrieved directly from MODELTEST (Posada & Crandall, 1998), and input as starting values.

RESULTS

Alignment of the 404 bp complete PCR product sequences collected from 58 of the 64 total salamanders sampled in this study (Table 1) revealed a total of ten haplotypes (the outgroup and nine ingroup haplotypes). No gaps were present in the alignment. Of the variable positions in the ingroup, 37 (68.5%) corresponded to third, five (9.3%) to second, and 12 (22.2%) to first codon positions. Of the variable third codon positions, 31 (83.8%) were transitions and six (16.2%) were transversions. Table 3 shows all pairwise distance comparisons (percent uncorrected sequence divergences) of these haplotypes, and these range from 0.0025 to 0.0099 within *P. cinereus*, from 0.0025 to 0.0049 within *P. shenandoah*, and from 0.099 to 0.1266 between species. All four *P. hubrichti* shared the same haplotype, while 26 *P. cinereus* segregated six, and 28 *P. shenandoah* segregated three haplotypes (Table 1).

The distribution of mtDNA haplotypes among the ingroup samples shows a pattern of high frequency or complete fixation of alleles diagnostic for each species (Table 1). At the Pinnacles, both species are characterized by single diagnostic haplotypes, while samples of both species at the Hawksbill Mountain and Stony Man Mountain isolates were polymorphic, but allelic composition was mutually exclusive between the two species at all three localities (Table 1). The Hawksbill Mountain site contains the highest allelic diversity within *P. cinereus*, with five alleles present, four of which are unique to this population. At Stony Man Mountain, only two alleles were found within the *P. cinereus* populations (alleles S8 and P9) and one of these is near fixation. Two of three *P. shenandoah* samples segregate for two alleles, one of which is unique to each population.

Estimated levels of genetic diversity with a fluctuating population size (Kuhner *et al*., 1998) give values approximately four times greater in *P. shenandoah* (θ = 0.0114) than in *P. cinereus* (θ = 0.00299). When the population size parameter is kept constant (Kuhner *et al*., 1995), the genetic diversity estimate for *P. shenandoah* (θ = 0.0022) is only slightly smaller than that of *P. cinereus* (θ = 0.0035).

The estimated parameters for the *Plethodon* sequences under the HKY85 model are as follows: Ti/Tv ratio = 5.3480, base frequencies A = .3487, C = .1252, G = .2262, T = .2999. Using these parameters in the ML analysis, a single tree was recovered with –lnL = 961.40 (Fig. 2). The NJ analysis also recovered a tree with an identical topology, with the trivial exception of very slight differences in branch support within the *P. cinereus* and *P. shenandoah* clades. All methods unambiguously recovered all ingroup haplotypes as reciprocally monophyletic for *P. cinereus* and *P. shenandoah*, respectively, with 100% bootstrap support (Fig. 2).

To evaluate the strength of support for this reciprocally monophyletic topology relative to an introgression

Table 3. Summary of pairwise distance values (uncorrected percent sequence divergence) for all non-identical haplotypes resolved in this study. Upper triangle contains numbers of transitions and transversions (Ti/Tv) for each pairwise comparison. Upper case letters following species names represent the three localities (H = Hawksbill, S = Stony Man, P = Pinnacles), and numbers identify distinct haplotypes.

	Haplotypes									
Taxa	1	2	3	4	5	6	7	8	9	10
1 *P. hubrichti*		36/4	35/5	37/4	42/8	41/8	41/9	41/8	41/8	42/8
2 *P. shenandoah* (H10)	0.0990		0/1	1/0	38/8	37/8	37/9	37/8	39/8	38/8
3 *P. shenandoah* (H2)	0.0990	0.0025		1/1	38/9	37/9	37/10	37/9	39/9	38/9
4 *P. shenandoah* (S10)	0.1015	0.0046	0.0025		39/8	38/8	38/9	38/8	40/8	39/8
5 *P. cinereus* (P9)	0.1240	0.1241	0.1216	0.1241		1/0	1/1	1/0	1/0	2/0
6 *P. cinereus* (S8)	0.1215	0.1266	0.1241	0.1266	0.0074		0/1	0/0	2/0	1/0
7 *P. cinereus* (H1)	0.1215	0.1216	0.1192	0.1216	0.0074	0.0099		0/1	2/1	1/1
8 *P. cinereus* (H8)	0.1191	0.1191	0.1167	0.1191	0.0050	0.0074	0.0025		2/0	1/0
9 *P. cinereus* (H2)	0.1216	0.1216	0.1191	0.1216	0.0025	0.0050	0.0050	0.0025		3/0
10 *P. cinereus* (H7)	0.1240	0.1241	0.1216	0.1241	0.0050	0.0025	0.0074	0.0050	0.0025	

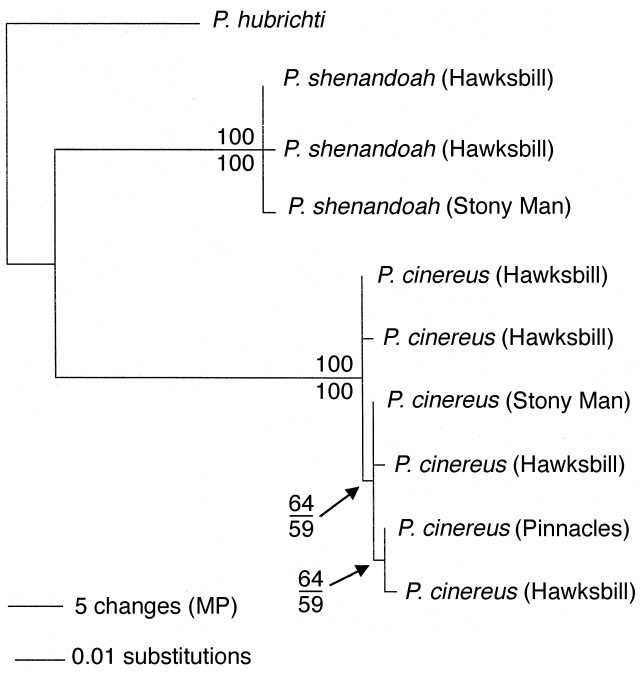

Fig. 2. Single tree recovered from the ML, MP and NJ searches of nine *Plethodon* mtDNA haplotypes using *P. hubrichti* as the outgroup. Bootstrap values for ML and NJ analyses are shown above and below the internal branches respectively. Bootstrap values for ML and MP are identical.

scenario, we performed statistical tests, using the method of Kishino & Hasegawa (1989), comparing the most parsimonious topology to alternatives in which (1) a single *P. cinereus* haplotype was constrained to monophyly with the *P. shenandoah* haplotypes (introgression of *P. cinereus* into *P. shenandoah*); (2) the reciprocal constraint was enforced (*P. shenandoah* introgression into *P. cinereus*). Table 4 shows that, under both ML and MP paired comparisons, the constraint trees are significantly less likely and parsimonious, respectively, than our best trees.

Table 4. Kishino–Hasegawa test values comparing constraint trees against the best tree for significance. Tree 1 constrains a single *P. cinereus* haplotype within the *P. shenandoah* clade and tree 2 constrains a single *P. shenandoah* haplotype within the *P. cinereus* clade. The third tree is the ML tree (Fig. 2). Trees 5 and 6 are MP constraint trees equivalent to trees 1 and 2 for ML, while tree 4 is the shortest MP tree.

Tree	–lnL	Difference in –lnL	Statistical difference	T	P*
1	961.40	37.38	11.31	3.31	0.0010
2	984.96	60.93	14.05	4.34	< 0.0001
3	924.03	best tree			
Tree	**Length**	**Difference**	**Statistical difference**	**T**	**P***
4	76	Shortest (best)			
5	94	18	4.15	4.34	<0.0001
6	103	27	5.22	5.17	<0.0001

DISCUSSION

Population structure of the *P. shenandoah* isolates

Questions of long-term historical fragmentation and hybridization between *P. shenandoah* and *P. cinereus* can be addressed using the data presented in Fig. 2, as well as palaeoclimatology records. Beginning in the Pleistocene approximately 2 million years ago, climate has interchanged between warm interglacial and cold glacial periods. During wetter, cooler periods, it is speculated that some plethodontids, such as *P. cinereus*, may have expanded their ranges substantially whereas other species (including *P. shenandoah*) were more confined (Highton, 1999; and pers. comm.).

As recently as 12,000 years ago, glaciers were present only 200 miles north and west of Shenandoah National Park, whose vegetation was dominated by spruce and fir rather than the oak–hickory–hemlock forests of today (Heatwole, 1997). It is possible that *P. shenandoah* was more widespread throughout the Blue Ridge Mountains in the past (Thurow, 1999; Highton, pers. comm.). However, factors such as changing climatic conditions and competitive interactions with *P. cinereus* may have restricted the range expansion or limited the distributions of *P. shenandoah* in recent times.

A pattern of long-term historical fragmentation would be evident if there were complete lineage sorting among the three populations of *P. shenandoah* (i.e., if each of these three populations was recovered as a monophyletic group). Our data show that this is not the case. Genealogical relationships among the *P. shenandoah* haplotypes as inferred by either of the two optimality criteria (ML and MP methods), as well as the NJ clustering of genetic distances, show that all three populations in Shenandoah National Park form a single monophyletic group (Fig. 2). Also, the mutational differences among the three haplotypes involve only one or two characters. Thus, there is no evidence for matrilineal genetic differentiation among the three isolates of *P. shenandoah*. These two lines of evidence suggest that the fragmentation of the three *P. shenandoah* isolates occurred relatively recently (post-Pleistocene), an interpretation consistent with the near absence of allozyme differentiation reported by Highton (1999). If these populations are completely isolated and small, and if the inbreeding effective sizes (N_{ei}) are small over the long term (Crandall, Posada & Vasco, 1999*b*), they may be vulnerable to extinction from any number of genetic, demographic or stochastic processes (Lande, 1988).

Gene flow could also account for the presence of shared alleles among the three isolates of *P. shenandoah* because this species can persist in the deeper soil outside the dry talus outcrops. However, they are normally competitively excluded from doing so by *P. cinereus* (Griffis & Jaeger, 1998). Given that *P. shenandoah* is physiologically more tolerant of dry conditions (Jaeger, 1971*b*), seasonal weather may occasionally favour movement of this species into the leaf litter, and some

animals might occasionally make the relatively short migration (~ 2 km) between isolates frequently enough to maintain the common alleles among all populations. One effective migrant per generation, especially among large populations, is usually sufficient to maintain genetic homogeneity among isolates (Mills & Allendorf, 1995).

Our estimate of genetic diversity (θ) is approximately four times greater in *P. shenandoah* (θ = 0.0114) than in *P. cinereus* (θ = 0.00299) under the assumption of fluctuating population size, suggesting that *P. shenandoah* is at least as demographically 'stable' as *P. cinereus* (with regard to N_{ei}). Under the restriction of constant population size, the diversity estimate for *P. shenandoah* (θ = 0.0022) is slightly smaller than that of *P. cinereus* (θ = 0.0035). The assumption of a constant population size for either of these species may not be a valid one, but many features of the biology of small species of *Plethodon* inhabiting eastern deciduous forests imply that N_{ei} will probably not be small for long periods of time, under normal circumstances. The seven currently recognized species of the *P. cinereus* group, which includes *P. shenandoah*, are often the most abundant vertebrates in eastern forests (Petranka, 1998; Highton, 1999). Some species of *Plethodon* are estimated to attain densities of several thousand animals per hectare (Burton & Likens, 1975; Hairston, 1987). Unless populations of *P. cinereus* and *P. shenandoah* deviate drastically from this pattern, or differ greatly in maturation time, generation time, adult sex ratio, or any other factors that determine effective population size (Nunney & Elam, 1994), the demographic data are consistent with genetic data in suggesting that population sizes of both species are usually large. In this case, infrequent gene flow among *P. shenandoah* isolates will hinder or prevent differentiation by genetic drift alone (see also Slatkin, 1993).

One major incongruence between the mtDNA and nuclear loci is the extremely high heterozygosity of the nuclear loci in the isolates of *P. shenandoah.* Highton's (1999) recent survey of allozyme variation across all species and multiple populations of the *P. cinereus* group revealed that average heterozygosities (H, the mean proportion of loci heterozygous per individual) were highest for the three *P. shenandoah* isolates ($\bar{H}$ = 0.11, range = 0.085 to 0.134; Table 1; see also Appendix 2 of Highton, 1999).

The presence of high nuclear heterozygosities coupled with limited mtDNA variability (except for *P. cinereus* at the Hawksbill site; Table 1) is consistent with a recent bottleneck in the ancestral *P. shenandoah* population. The mitochondrial locus is expected to show greater sensitivity to loss of alleles by drift in small effective population sizes (Birky, Marayuma & Fuerst, 1983; Birky, Fuerst & Marayuma, 1989), while the relative insensitivity of Mendelian loci to short-term bottlenecks (Nei, Marayuma & Chakraborty, 1975) can explain the absence of bottleneck effects on allozyme heterozygosity. Collectively, these observations are consistent with an interpretation of recent isolation of *P. shenandoah* populations coupled with a large N_{ei}, albeit possibly accompanied by a historical population bottleneck of short duration. Although higher allozyme heterozygosity within the Hawksbill and Stony Man Mountain sites may be due to past or ongoing hybridization (Highton, 1999), evidence for extensive introgression of *P. cinereus* alleles into *P. shenandoah* is lacking, and alternative interpretations must be considered.

Hybridization and genetic integrity of the *P. shenandoah* isolates

The second issue of concern is that of hybridization, and the possible introgression of *P. cinereus* alleles into *P. shenandoah* populations. Highton & Worthington (1967) reported evidence of historical or possibly ongoing *P. cinereus* × *P. shenandoah* hybridization at the northeastern edge of the Hawksbill Mountain isolate. Hybridization may also be occurring between *P. cinereus* and the newly discovered populations of *P. shenandoah* to the southwest (Thurow, 1999). Hybridization was inferred because some individuals showed characteristics (e.g., dorsal stripe width, extent of ventral pigmentation) intermediate between the two species. Highton & Worthington (1967) originally suggested that Hawksbill Mountain is the only site where hybridization occurs. Although allozyme data confirm this (unpublished data of A. Wynn & R. Highton; cited in Highton, 1999), Thurow (1999) has now described morphological intermediates at Pinnacles and Stony Man, as well as in three new populations from the southwestern Virginia Blue Ridge area. If, in fact, hybridization is occurring between the two species then the concern is that *P. shenandoah* may eventually be genetically swamped by an influx of alleles from the enormous populations of *P. cinereus* that surround the *P. shenandoah* isolates. However, Highton & Worthington (1967) stated that introgression at the periphery of the Hawksbill site was asymmetrical in the direction of *P. shenandoah* into *P. cinereus.* They reported (1) individuals of *P. cinereus* within 0.25 miles of the Hawksbill *P. shenandoah* isolate with morphological characters diagnostic for *P. shenandoah*, and (2) there was no morphological evidence of *P. cinereus* introgression into *P. shenandoah* populations in any of the three isolates. If this is true, then there is little concern for eventual genetic swamping of *P. shenandoah.*

Although we cannot rule out the possibility that some hybridization may occur on a restricted scale, our data (Fig. 2) indicate that both species have been isolated for a long time, and genetically there is no introgression of mtDNA alleles in either direction at any of the three isolated *P. shenandoah* localities. The individuals sampled for this study were unambiguously identified as representatives of their respective species and no morphological intermediates were included. If there is extensive hybridization occurring at the periphery of the range of *P. shenandoah* at Hawksbill, then the hybrids and/or backcrosses appear to be sufficiently restricted to the narrow zones of parapatric contact, so as to preclude

gene flow into the core area of the main population of *P. shenandoah*. In order to determine if there is a genetic basis (i.e., hybridization) for the morphological intermediates reported by Highton & Worthington (1967) and Thurow (1999), a detailed transect sampling design would have to be established through the zone of admixture, and classes of individuals – pure *P. cinereus*, pure *P. shenandoah*, and *P. cinereus* × *P. shenandoah* 'hybrids' – would need to be unambiguously scored for multiple diagnostic genetic markers (Arnold, 1997). It is likely that the morphological intermediates (Highton & Worthington, 1967; Thurow, 1999) are in fact hybrids, but available distributional evidence so far suggests that, at least at Hawksbill and Stony Man, 'the two species usually overlap only in a very narrow contact zone, sometimes only a few meters in width' (Highton, 1999: 49–50). Our evidence for a lack of extensive introgression is based on data from a single genetic marker and small sample sizes taken from the 'core' areas of *P. shenandoah*.

Sample sizes needed to reliably detect allele frequency differences at a polymorphic locus are inversely related to the magnitude of frequency difference between populations (Baverstock & Moritz, 1996), and in this case, mtDNA haplotype differences appear to be absolute. While sample sizes at any single locality are small, combining localities within species provides sufficiently large samples (Table 1; $n = 26$ for *P. cinereus*, $n = 28$ for *P. shenandoah*) to reject H_0 (no frequency difference between samples) at $P < 0.20$, with a power of 80%, given a 0.90 allele frequency difference between the two samples (Table 1, Baverstock & Moritz, 1996; see also Wiens & Servedio, 2000). While this is not a conventional probability level, we argue that given unknown, but possibly beneficial (Grant & Grant, 1992; Rhymer & Simberloff, 1996) effects of limited trans-species introgression, this is an acceptable type-I error level for rejection of H_0.

A second argument in favour of limited *P. cinereus* × *P. shenandoah* introgression comes from the allozyme allele frequencies. We present a summary of these data from Highton (1999, Appendix II) for polymorphic protein loci which are potentially informative on the issue of *P. cinereus* × *P. shenandoah* introgression (Table 1), and offer the following observations. First, for some loci the same alleles are shared across species (IDH-1, IDH-2 and AAT-1). This pattern could be due to gene flow across a hybrid zone, or simply reflect a shared ancestral polymorphism at each locus. Second, and more importantly, all other loci in Table 1 show patterns in which alleles at high frequency in one species are absent from the other. In *P. cinereus*, for example, the allele Est-β(n) occurs in high frequency, but is absent from all samples of *P. shenandoah*. The same pattern is evident at the TRF locus for which high frequency diagnostic alleles in each species (TRF(g) in *P. cinereus*, and TRF(d,h) in *P. shenandoah*) fail to appear in the other. Similarly, diagnostic *P. shenandoah* alleles (α-GPD(c), PGDH(a), and many alleles at EST-β) are absent from the *P. cinereus* sample. Given the larger sample sizes used by Highton (1999), and the levels of polymorphism at many of these loci (Table 1), the above pattern offers weak evidence for very limited introgression at best, and other interpretations cannot be unambiguously ruled out. We argue that in the face of no prior statistical or genetic evidence to support large-scale hybridization, and the absence of detailed transect studies that bridge the geographic scale between the sampling design of this study and that of Highton & Worthington (1967), available evidence shows a lack of extensive introgression of alleles in either direction. Future work should include genetic surveys of the populations recently described by Thurow (1999), and microgeographic studies of hybridization in regions of parapatric contact between *P. cinereus* and *P. shenandoah*.

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