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RESEARCH ARTICLE

Assessing the genetic consequences of habitat fragmentation on the federally threatened cheat mountain salamander (*Plethodon nettingi*): a comparative, multi-locus approach

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Abstract
Comparative population genetic studies of closely related taxa provide a powerful framework for evaluating if and to what degree a species of conservation concern has been negatively impacted by factors such as habitat fragmentation, decreased population connectivity, inbreeding and genetic drift. In this study, we take advantage of a paired sampling strategy to compare the population genetics of the geographically restricted, federally threatened Cheat Mountain salamander (*Plethodon nettingi*) to those of its partially sympatric, but much more widely distributed congener, the red-backed salamander (*P. cinereus*), where the two species overlap in the Appalachian mountains of West Virginia. Mitochondrial DNA haplotype and nucleotide diversity were lower in *P. nettingi*, as were a variety of metrics of nuclear genetic diversity estimated from microsatellite data. Population differentiation and structuring were greater in *P. nettingi*, suggesting reduced gene flow following fragmentation. Significant inbreeding and evidence of recent population bottlenecks were also seen in *P. nettingi* and estimated population sizes were smaller. Estimates of contemporary gene flow, as measured through kinship, also showed more restricted gene flow in *P. nettingi*. Overall, our comparative study provides strong evidence that the small and highly fragmented nature of its geographic distribution has resulted in a suite of negative genetic consequences for the federally threatened Cheat Mountain salamander. Management efforts aimed at enhancing the genetic health and long-term viability of this species should focus on increasing population connectivity through establishment of forest habitat corridors where possible and exploring the potential merits of translocations.

Keywords Appalachian · Gene flow · Genetic diversity · Genetic structure · Microsatellites

Introduction

Human activities over the past several hundred years have caused large-scale impacts on many ecosystems, greatly accelerating rates of population declines. In addition to changes in habitat, the loss of genetic diversity due to reduced population sizes and reduced population connectivity has become a major conservation issue for many threatened and endangered species (Avise 1995; Kuo and Janzen 2004; Frankham et al. 2017; Ralls et al. 2018). For forest-associated amphibians in particular, forest loss and fragmentation are expected to strongly influence landscape connectivity, potentially altering the dispersal patterns of organisms that inhabit fragmented habitats and, in turn, gene flow and genetic diversity of populations (Almeida-Gomes and Rocha 2014; Vallan 2000). Loss of genetic diversity and increased inbreeding also can reduce a population's adaptive response to climate change (Jordan et al. 2009; Frankham et al. 2012). Thus, in the absence of focused conservation actions, mountain-top species composed of small and highly fragmented populations are likely to face a variety of demographic and genetic challenges to their long-term persistence (Templeton et al. 2011).

The Appalachian mountains are a biodiversity hotspot for plethodontid salamanders, the largest genus of salamanders in North America with > 50 recognized species (Rissler and Smith 2010; Bayer et al. 2012). Many species of *Plethodon* have limited dispersal ability and exhibit strong site fidelity, which can promote isolation and local speciation, resulting

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in species endemic to a single mountaintop or mountain range (Highton 1995; Carpenter et al. 2001; Wiens et al. 2006; Cabe et al. 2007; Marsh et al. 2008; Kozak and Wiens 2010). Montane endemics can be especially sensitive to environmental perturbations due to limited potential for range shifts and can be particularly susceptible to reductions in genetic variation via genetic drift in their small, isolated populations (Rissler and Smith 2010; La Sorte and Jetz 2010; Bayer et al. 2012; Highton et al. 2012).

The Cheat Mountain salamander, *Plethodon nettingi*, is a small, woodland salamander found only in the high-elevation red spruce (*Picea rubens*) and mixed red spruce-hardwood forests of the central Appalachian mountains of northeastern West Virginia (Fig. 1). They are tied to cool, moist microhabitats (particularly those found under rocks and coarse woody debris) of the forest floor and presumably complete their entire life cycle within these forests (Heatwole 1962; Green and Pauly 1987, Herbeck and Larsen 1999; Dillard et al. 2008a). Although the specific movement patterns of *P. nettingi* are poorly known, they are thought to be similar to those of their congener, *P. cinereus*, a species in which individuals typically have a small home range and disperse only short distances (i.e., < 5 m) from their natal site over the course of their lifetime (Kleeberger and Werner 1982; Marsh et al. 2004; Marsh et al. 2007; Liebgold et al. 2011; Jaeger et al. 2016; but see Marsh et al. 2004). Due to Pleistocene forest dynamics (Soltis et al. 2006; Hewitt 2011) and more recent episodes of intensive logging and intense fires in the late 1800s/early 1900s, the distribution of the high-elevation red spruce forest in which *P. nettingi* lives is extremely fragmented in this region, existing as a series of high-elevation habitat islands on disjunct mountaintops (Pauley 2008a, b). Furthermore, the current geographic range of *P. nettingi* is estimated to span only 92 km north to south and 13 km east to west with approximately 80 distinct populations of *P. nettingi* identified to date (Pauley 2008a, b; USFWS 2009).

Based in large part on its limited and highly fragmented geographic distribution, *P. nettingi* was listed as federally threatened under the U.S. Endangered Species Act in 1989 (Federal Register 1989) and remains so listed today. Many threats to *P. nettingi* still exist, including road barriers, climate change, pathogens, and interspecific competition with other salamanders (Pauley and Clovis 1980; Greathouse and Pauley 2008; Pauley 2008a, b; Greathouse 2011). *P. nettingi*'s presumably low dispersal ability and highly fragmented distribution suggest that inter-population exchange of individuals, and thus genes, is potentially quite limited in this species (USFWS 1991, 2009). Together, these factors raise serious questions about the current genetic health of *P. nettingi* and the long-term viability of this species. However, management actions have been hindered by a lack of any previous population genetic studies on *P. nettingi*.

Unfortunately, studying gene flow in taxa such as *P. nettingi* can be challenging due to their low abundance and constraints

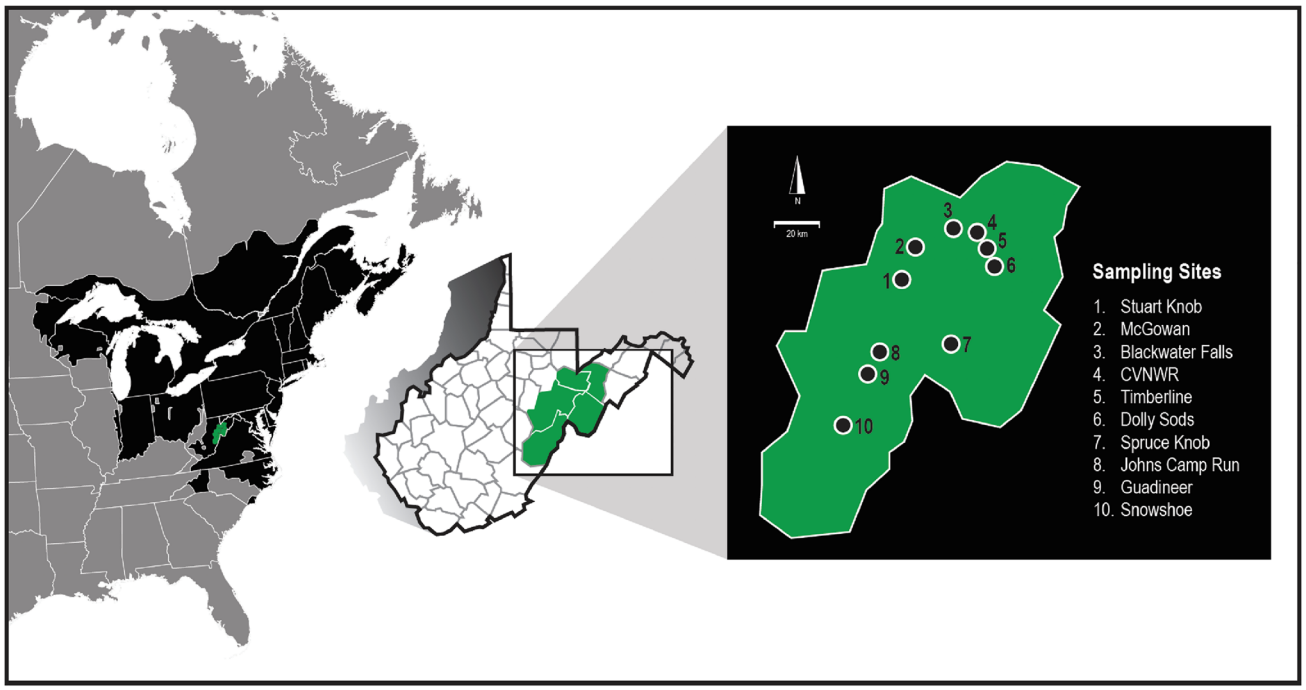

Fig. 1 Distribution of *P. cinereus* (black) and distribution of *P. nettingi* (green). The green area represents the five-county region in West Virginia in which *P. nettingi* is found. All sampling was performed within the known distribution of *P. nettingi*. Specific sampling sites are shown in panel at right. (Color figure online)

associated with obtaining representative samples from their small, patchy, and in the case of *P. nettingi*, relatively remote geographic distribution. Furthermore, in population genetic studies of threatened or endangered species, management conclusions must often be made based on relatively few individuals or populations (Lowe and Allendorf 2010; Cameron et al. 2019). As such, obtaining comparative data on the population connectivity and genetic diversity of a closely related species that is more abundant and geographically widespread, but ecologically similar to a rare, threatened species, can aid conservation biologists in understanding how population dynamics and associated genetic parameters are altered in fragmented landscapes. In turn, this type of comparative approach can facilitate the development of more effective management strategies.

The red-backed salamander, *P. cinereus,* is an excellent model for evaluating the conservation genetics of *P. nettingi* as the two species overlap geographically in the Appalachian mountains of eastern West Virginia (Fig. 1). However, the red-backed salamander is much more abundant and widely distributed than the Cheat Mountain salamander and is not considered to be a species of conservation concern (Heatwole 1962; Adams et al. 2007; Cabe et al. 2007; Pauley 2008a). Much of the current geographic range of *P. cinereus* (Fig. 1) lies north of the southernmost extent of the Laurentide ice sheet during the last (Wisconsinin) glacial maximum, and phylogeographic analyses support a post-glacial northern expansion in this species, most likely from an Appalachian refugium (Radomski et al. 2020). Several previous genetic studies on *P. cinereus* (*i.e.,* Gibbs 1998; Jordan et al. 2009; Wilk et al. 2020) from various parts of its range have shown relatively weak population differentiation, low levels of inbreeding and no evidence of significant historical bottlenecks.

Here, we use a multi-locus and multi-species comparative approach to conduct the first conservation genetics study of the federally threatened Cheat Mountain salamander. Our aims were to (a) estimate overall levels of intraspecific genetic variation, elucidate geographic patterns of population genetic structure and infer historical and contemporary patterns of gene flow in *P. nettingi*, (b) to compare these results to those obtained from the red-backed salamander (*P. cinereus*), a closely related congener of *P. nettingi* and (c) to use the results of this comparative approach to provide new genetic-based insights to help inform the management and conservation of the Cheat Mountain salamander.

Methods

Sample collection and preparation

We obtained samples for genetic analysis from the West Virginia Division of Natural Resources (WVDNR) and United States Fish and Wildlife Service (USFWS). Samples consisted of tail tissue clips, preserved in ethanol, from 108 individuals of *P. nettingi* and 100 individuals of *P. cinereus*, collected between 2008 and 2011 from 11 representative localities from throughout the range of overlap between the two species in northeastern West Virginia; these localities represent a large portion of the total range of *P. nettingi* (Fig. 1). We extracted genomic DNA using the QIAGEN DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's standard protocol for DNA isolation from animal tissue and conducted all laboratory analyses at the University of North Carolina Wilmington (UNCW).

Mitochondrial DNA

We amplified and sequenced a 723 base-pair (bp) segment of the mtDNA ND4 gene for all samples using the primers ND4(F) and Ephist(R) from Wiens et al. (2006). PCR amplification followed the protocol of Shepard and Burbrink (2009). Clean-up and sequencing of PCR products was performed at the High Throughput Genomics Center (University of Washington, Seattle, WA, USA). All individuals were first sequenced using the Ephist(R) primer only, and all unique haplotypes were subsequently sequenced using the ND4(F) primer as well.

We visually inspected mtDNA sequences for errors and multiple peaks, trimmed, and aligned them using SEQUENCHER v4.9 (Gene Codes Corporation, Ann Arbor, MI). All mtDNA sequences were deposited to GenBank (accession numbers KX061194-KX061293 for *P. cinereus* and KX061294-KX061401 for *P. nettingi*). We used MODELTEST v3.7 (Posada and Crandall 1998) to infer a best-fit model of nucleotide substitution for the mtDNA data set for each species; this model was then used in subsequent phylogenetic analyses. We used PAUP v.4.0a136 (Swofford 2003) to infer a haplotype tree for each species under the best-fit model using a maximum likelihood (ML) approach with 1000 ML bootstrap replicates to assess nodal support. We also used MRBAYES v3.2.0 (Huelsenbeck and Ronquist 2001) to infer a haplotype tree for each species using a Bayesian approach. For each MCMC run, we used a burn-in of 50,000 with a sampling frequency of 5000 generations and a run length of 1,000,000 generations. We used three congeneric species (*P. hoffmani, P. virginia*, and *P. hubrichti*) as outgroups for the *P. nettingi* analyses, and *P. hoffmani*, *P. virginia*, and *P. shenandoah* as outgroups for the *P. cinereus* analyses. We used TCS v1.21 (Clement et al. 2000) to create haplotype networks with a 95% parsimony connection limit of nine nucleotide changes (Templeton et al. 1992). We used DNASP v5.10.01 (Librado and Rozas 2009) to evaluate within-group mtDNA nucleotide diversity (π; Nei 1987)

and the average number of nucleotide differences between sequences (*k*; Tajima 1983).

Microsatellites

We genotyped individuals at eight polymorphic microsatellite loci using previously developed *Plethodon* primers (Connors and Cabe 2003; Cabe et al. 2007; Supplementary Information Table S1). We amplified microsatellite loci following the protocols outlined in Connors and Cabe (2003); Cabe et al. (2007). We mixed 0.5 µL of PCR product with 0.2 µL GeneScan-500 ROX size standard and 9.3 µL Hi-Di Formamide (Applied Biosystems, Foster City, CA, USA) for genotyping on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems) at the UNCW Center for Marine Science Core Genetic Laboratory. We scored fragments using STRAND v2.3.69 (Toonen and Hughes 2001).

We tested for departure from Hardy–Weinberg equilibrium (HWE) within each locality by locus using GENEPOP v4.2 (Raymond and Rousset 1995). We ran MICRO-CHECKER v2.2.3 to determine whether any deviations from HWE were due to null alleles or large allele drop-out, as well as to check for stuttering (Van Oosterhout et al. 2004). We calculated standard diversity indices, tests for linkage disequilibrium (LD), inbreeding coefficients (F_{IS}), and observed and expected heterozygosity (H_O and H_E) in ARLEQUIN v3.5 (Excoffier and Lischer 2010). We calculated allelic richness, rarefied to the minimum number of individuals in a sampling location, using the PopGenReport package in R (Gruber and Adamack 2015).

We calculated all pairwise values of population differentiation using Weir and Cockerham's *F*-statistics (Weir and Cockerham 1984) and tested for significance with 10,000 permutations of the data. We conducted Mantel tests in IBDWS v3.2.3 (Jensen et al. 2005) to test for a correlation between genetic and geographic distance among localities. The geographic distance (in km) between locations was the shortest straight-line path connecting those points. We used STRUCTURE v2.3.4 to detect genetic structure and assign individuals to clusters (Pritchard et al. 2000). We assessed the number of genetic clusters (K) within each species for values of K ranging from 1 to 10 using the admixture model with allelic frequencies correlated among populations and including prior population information. We ran ten Bayesian Markov chain Monte Carlo (MCMC) searches of 1,000,000 steps with a 100,000-step burn-in. STRUCTURE results were collated and ΔK was computed via the Evanno method (Evanno et al. 2005) using STRUCTURE HARVESTER web version 0.6.94 (Earl and VonHoldt 2012), to find the value of K that best fit the observed distribution of genotypes. Because STRUCTURE elucidates the highest level of genetic structure, it is possible to test for hierarchical structure by running each identified cluster separately. We evaluated all clusters (K) identified in the initial analysis again separately to search for substructure. To further partition genetic variance within and among clusters identified by STRUCTURE, we implemented a hierarchical analysis of molecular variance (AMOVA) in ARLEQUIN.

To test for population bottlenecks, we used BOTTLENECK v1.2.02 (Piry et al. 1999). The mode-shift test can detect bottlenecks within the past 25–50 generations and the Wilcoxon's sign-rank test can detect older bottlenecks up to 250 generations in the past (Cornuet and Luikart 1996). We chose the two-phase model (TPM; Di Rienzo et al. 1994) because it is more appropriate than the strict one-step stepwise mutation model (SMM; Ohta and Kimura 1973) for microsatellite data (Piry et al. 1999). We set the parameters for TPM to 95% single-step mutations and 5% multiple step mutations and set the variance among multiple steps to 12% as suggested by Piry et al. (1999). Because small sample sizes can lead to low statistical power in detecting bottlenecks, we also ran tests on the population clusters identified by STRUCTURE. We used LAMARC v1.2.1 (Kuhner et al. 2006) to estimate unidirectional migration among the populations. We estimated theta and *M* using 10 initial chains with 2000 genealogies sampled and two final chains with 20,000 genealogies sampled. Theta is defined as $4N_e\mu$ for diploid organisms, where N_e is the effective population size and µ is the mutation rate. Migration is expressed as *M* migrants per generation between populations.

Finally, to investigate spatial patterns of family structure, we calculated kinship coefficients (Loiselle et al. 1995), which are based on the relative probability of two alleles being identical by descent between each pair of individuals, using GENODIVE (Meirmans and Van Tienderen 2004). To determine whether kin were more likely to be clustered in the same sampling location, we used the PERMANOVA + 1.0.2 software add-on in PRIMER7 (Clarke and Gorley 2006) to conduct a 1-way ANOVA on kinship coefficients following the approach of Iacchei et al. (2013). To further investigate relationships among salamanders within and among sampling locations, we binned individuals according to the following levels of kinship *(k)*: 'nearly identical', $0.57 > k > 0.375$; 'full siblings', $0.375 > k > 0.1875$; 'half siblings', $0.1875 > k > 0.09375$; and 'quarter siblings', $0.09375 > k > 0.047$. Here the bounds represent the midpoints between coancestry coefficients (Loiselle et al. 1995; Iacchei et al. 2013). We also conducted a permutation test where the observed number of closely related individuals within locations was compared to a null distribution of kinship coefficients generated by randomly assigning salamanders to localities (Iacchei et al. 2013). For this analysis, we only included sampling locations with > 5 individuals.

Results

Mitochondrial DNA

Analyses of the mtDNA ND4 sequence data recovered a total of six variable sites and six unique haplotypes for *P. nettingi*, and 30 variable sites and 14 unique haplotypes for *P. cinereus* (Fig. 2). We found that maximal levels of intraspecific mtDNA nucleotide diversity (π) were approximately three times higher in *P. cinereus* compared to *P. nettingi* (0.00696 vs. 0.00190, respectively) and the average number of nucleotide differences between sequences (k) was nearly four times higher in *P. cinereus* than in *P. nettingi* (3.707 vs. 0.940, respectively).

MODELTEST selected the TVM + I model for *P. nettingi* and the HKY + I model for *P. cinereus.* A single best ML tree, and a consensus Bayesian tree, were recovered for each species using PAUP and MRBAYES, respectively. All *P. nettingi* haplotypes were monophyletic with respect to the outgroups with ML bootstrap and Bayesian posterior probability (BPP) scores of 97 and 1.0, respectively. However, there was little phylogenetic structuring and no significant support for internal nodes within the *P. nettingi* clade (Supplementary Information Fig. S1). Monophyly of the *P. cinereus* haplotypes was well supported with ML bootstrap and BPP scores of 82 and 0.99, respectively. Within the *P. cinereus* clade, there was more phylogenetic structuring than that observed in the *P. nettingi* tree and several genetic groupings exhibited strong nodal support (Supplementary Information Fig. S2).

For both *P. nettingi* and *P. cinereus*, we produced haplotype networks using TCS with a 95% parsimony connection limit of nine nucleotide changes. The *P. nettingi* network showed all six haplotypes joined in one network (Fig. 2a) and the *P. cinereus* network showed all 14 haplotypes joined in one network (Fig. 2b). *P. nettingi* displayed a clear spatial arrangement of haplotypes between eastern and western localities, while *P. cinereus* showed much greater haplotype diversity within, and less structure across, localities (Figs. 3a, b).

Microsatellites

A global test for the presence of null alleles in MICRO-CHECKER revealed that only locus PcJX24 in *P. nettingi* showed potential evidence of null alleles, although the frequency was low. There were significant deviations from HWE in at most two loci at each sampling location (Table 1), with most locations showing no significant deviations in any loci. The percentage of loci in LD ranged from 0 to 11% across all locations and species, with no specific patterns emerging among loci. We found that the mean number of alleles per locus in *P. nettingi* ranged from 2.7 to 5.7 and was lower than in *P. cinereus* (range: 3.6–7.9). The pattern was similar in measures of allelic richness as well (Table 1). Overall, we found that values of H_O and H_E were lower in *P. nettingi* than in *P. cinereus* and significant F_{IS} estimates were detected in 50% of the locations sampled for *P. nettingi*, as compared to 29% of those sampled for *P. cinereus.*

We found that pairwise comparisons of genetic differentiation were significant in all cases in both species (Supplementary Information Table S2). However, overall F_{ST} values were larger in *P. nettingi* than in *P. cinereus* indicating a greater level of population differentiation in the former. Genetic distance was significantly correlated with geographic distance in *P. nettingi* ($r^2 = 0.25$, $P < 0.05$) but not in *P. cinereus* ($r^2 = 0.03$, $P > 0.05$; Fig. 4).

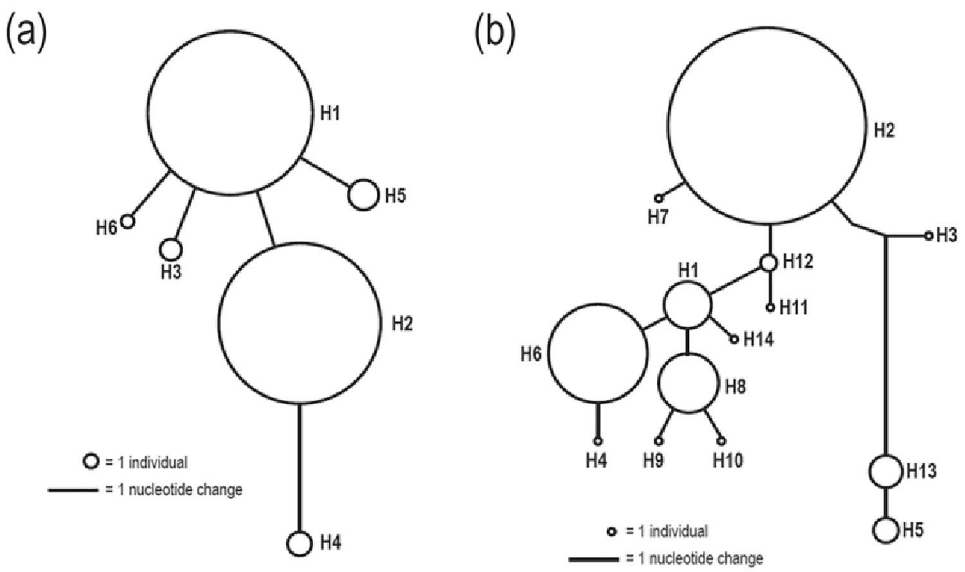

Fig. 2 MtDNA haplotype networks and geographic distribution of (**a**) *P. nettingi* and (**b**) *P. cinereus* haplotypes. Each circle represents a unique haplotype, and the size of each circle is proportional to the number of individuals

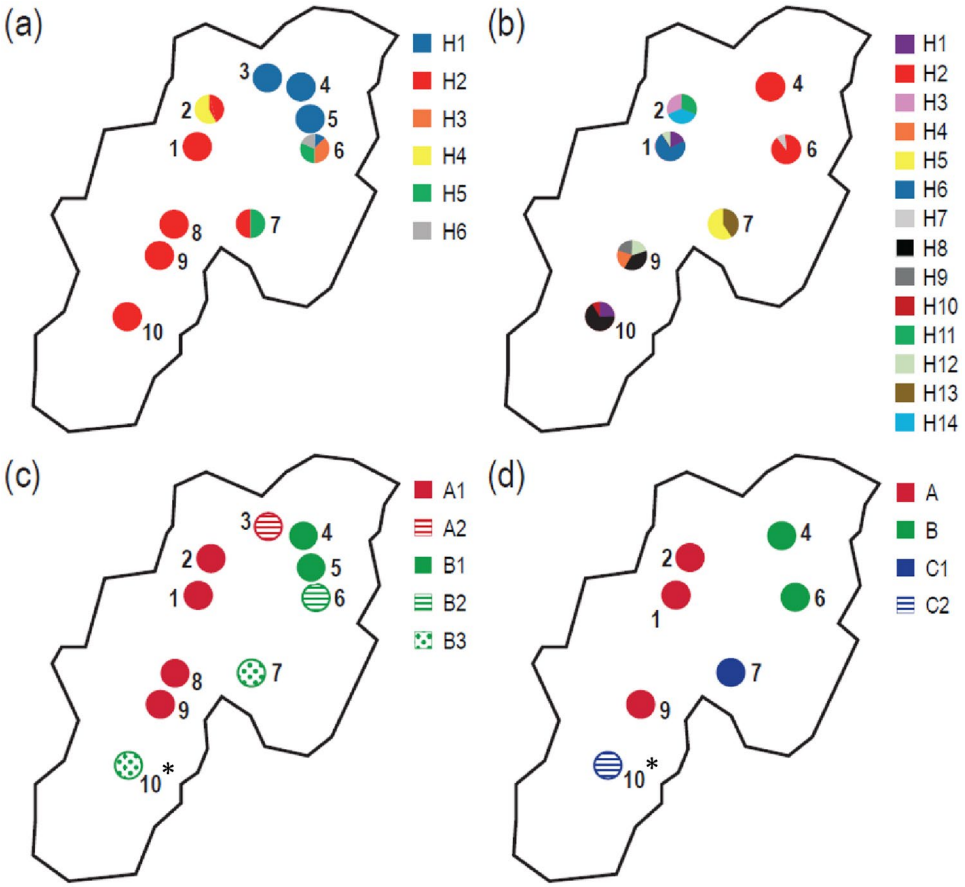

Fig. 3 Geographic distribution of mtDNA haplotypes for (**a**) *P. nettingi* (n = 6) and (**b**) *P. cinereus* (n = 14) across the sampled area in west Virginia; each color represents a unique haplotype. Geographic distribution of STRUCTURE population clusters for (**c**) *P. nettingi* (n = 5) and (**d**) *P. cinereus* (n = 4). Each color represents a unique genetic cluster; substructure is represented by dashed or dotted patterns. * indicates the locality (Snowshoe) where individuals were not unambiguously assigned to a cluster. (Color figure online)

The clustering analysis performed by STRUCTURE identified a peak at K = 2 for *P. nettingi*, which suggested two genetic clusters, as well as an additional peak at K = 4, which potentially indicates substructure within the two main clusters. The substructure analysis of *P. nettingi* showed further population structure, with two subgroups contained in cluster A and three subgroups contained in cluster B (Fig. 3c). Individuals from a given locality were unambiguously assigned to the same cluster (except for the individuals from Snowshoe): cluster A1 contained Stuart Knob, McGowan, John's Camp, and Guadineer, cluster A2 contained Blackwater, cluster B1 contained CVNWR and Timberline, cluster B2 contained Dolly Sods, and cluster B3 contained Spruce Knob and Snowshoe (Supplementary Information Fig. S3).

For *P. cinereus*, we identified a peak at K = 3, with substructure analysis identifying two subgroups within cluster C (Fig. 3d). Individuals from a given locality were unambiguously assigned to the same cluster (except for the individuals from Snowshoe): cluster A contained Stuart Knob, McGowan, and Guadineer, cluster B contained Dolly Sods and CVNWR, cluster C1 contained Spruce Knob and cluster C2 contained Snowshoe (Supplementary Information Fig. S4). Using the above information, the AMOVA analyses returned significant hierarchical population structuring among clusters and among locations within clusters in both species (Table 2).

We found no support for older bottlenecks in any locality or genetic cluster in *P. cinereus.* The mode-shift tests found shifted distributions in McGowan and Guadineer, though both localities had very small sample sizes (n = 3 and 4, respectively) and results should be interpreted with caution (Table 3). In *P. nettingi*, three localities showed signatures of heterozygote excess (Blackwater, Timberline, and Dolly Sods) and shifted distributions (Timberline, Spruce Knob, and Snowshoe; Table 3). Similar to *P. cinereus*, we found no support for recent or older bottlenecks in any genetic cluster in *P. nettingi.*

Theta estimates were higher in *P. cinereus* than in *P. nettingi* (mean = 1.29 vs. 2.89 for *P. nettingi* and *P. cinereus*, respectively; Supplementary Information Table S3). The unidirectional migration estimates were largely similar among locations in both species, suggesting that any reductions in gene flow would likely be recent. Indeed, kinship coefficients were significantly greater for within-location than among-location comparisons (pseudo-$F_{9, 107} = 1.443$, $p < 0.001$ and pseudo-$F_{6, 99} = 1.443$, $p < 0.001$, for *P. nettingi*

Table 1 Summary statistics for each *P. nettingi and P. cinereus* location averaged over all 8 microsatellites

Locality	n	A	AR	F_{IS}	H_O	H_E	% out of HWE	% loci in LD	*k*
Stuart knob									
nettingi	18	5.7	3.1	**0.155**	0.53	0.63	12.5	7.1	0.094
cinereus	27	7.9	3.4	**0.119**	0.59	0.67	25	0	0.117
McGowan									
nettingi	13	4.8	3.2	**0.132**	0.47	0.53	12.5	3.6	0.071
cinereus	3	3.6	3.3	**0.200**	0.62	0.74	0	0	0.141
Blackwater									
nettingi	12	4.7	2.9	0.014	0.58	0.59	0	0	0.324
CVNWR									
nettingi	20	4.8	2.8	0.085	0.45	0.49	0	7.1	0.176
cinereus	29	7.6	3.4	0.058	0.69	0.73	25	10.7	0.114
Timberline									
nettingi	10	4.0	2.9	**0.163**	0.53	0.63	12.5	3.6	0.155
Dolly sods									
nettingi	11	4.3	3.3	**0.147**	0.53	0.62	12.5	7.1	0.152
cinereus	10	6.0	3.5	0.079	0.69	0.74	0	7.1	0.149
Spruce knob									
nettingi	10	5.0	3.5	0.061	0.59	0.62	12.5	10.7	0.163
cinereus	10	4.5	2.9	− 0.022	0.64	0.62	0	3.6	0.299
John's camp									
nettingi	4	3.4	2.5	**0.294**	0.50	0.68	12.5	7.1	0.301
Guadineer									
nettingi	7	3.6	2.7	**0.120**	0.45	0.51	0	7.1	0.229
cinereus	5	3.6	2.8	0.116	0.50	0.56	0	0	0.248
Snowshoe									
nettingi	4	2.7	2.3	− 0.054	0.54	0.52	0	0	0.387
cinereus	15	7.4	3.5	0.064	0.70	0.75	12.5	0	0.113

n sample size, *A* mean number of alleles per locus, *AR* mean allelic richness, F_{IS} mean inbreeding coefficient, H_O observed heterozygosity, H_E expected heterozygosity, *LD* percentage of loci out of HWE, percentage of loci pairs in linkage disequilibrium, *k* mean kinship coefficient

Bold: significant at $p < 0.05$

and *P. cinereus*, respectively), indicating that the majority of kin comparisons were between individuals from the same locality. We also found that the mean kinship among salamanders within locations for *P. nettingi* was 0.163 ± 0.151, which was significantly higher than the mean kinship among salamanders from different locations (-0.021 ± 0.119). Within-location kinship in *P. nettingi* was also significantly greater than that for *P. cinereus* (0.124 ± 0.112). In fact, there were more kin groupings than would be expected by chance in *P. nettingi* than in *P. cinereus*, as well as fewer unrelated individuals than should be expected in *P. nettingi* (Fig. 5).

Discussion

With > 40% of amphibian species estimated to be threatened with extinction (IUCN 2021), the loss of amphibian biodiversity is clearly a global conservation crisis. Previous multi-locus population genetic studies (e.g. Blois and Arbogast 2006; Chavez et al. 2014; Rhoads et al. 2017; Pröhl et al. 2021) have highlighted the importance of natural and anthropogenic forest fragmentation in shaping the genetic architecture of forest-obligate species. As such, we might expect to see strong population structuring in both *P. nettingi* and its partially sympatric congener, *P. cinereus,* across this region of the central Appalachian mountains. However, the much smaller and patchier contemporary geographic distribution of *P. nettingi* (Fig. 1), along with its tighter affinity to the high-elevation red spruce forest of the region, suggests this species may exhibit markedly lower levels of genetic variation and higher levels of inbreeding than *P. cinereus.*

Our results are consistent with the above prediction. For example, we found reduced genetic variation in *P. nettingi* compared to *P. cinereus* as reflected in a smaller number of variable mtDNA nucleotide sites and unique haplotypes

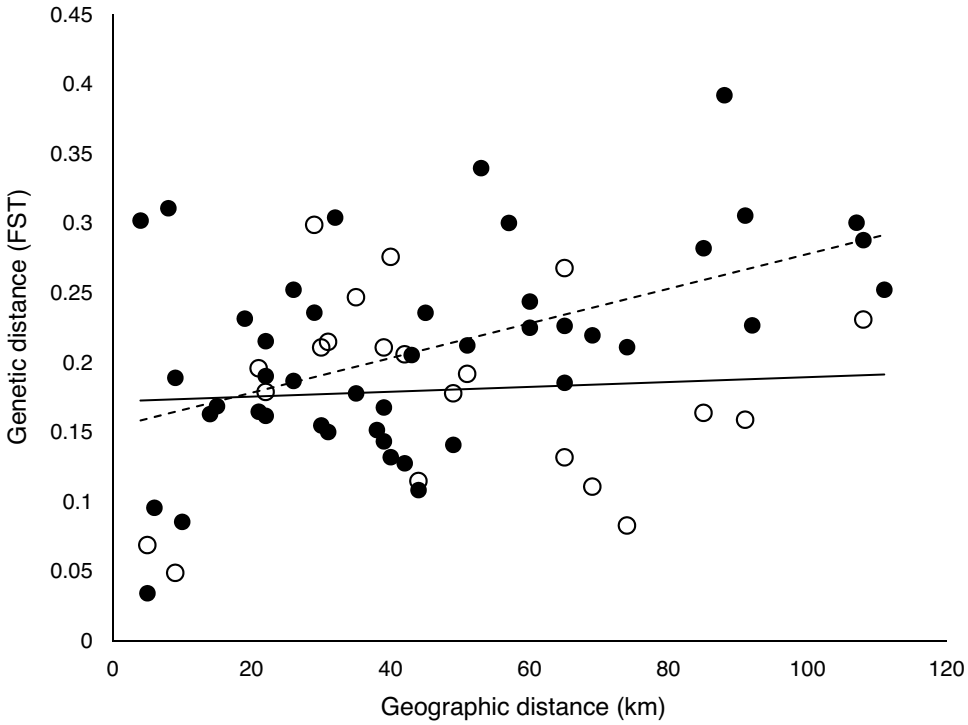

Fig. 4 Relationship between geographic (km) and genetic (F_{ST}) distance between localities for *P. nettingi* (solid circles) and *P. cinereus* (open circles). The correlation between distance matrices was only significant for *P. nettingi* (dashed line: $r^2 = 0.25$, $P < 0.05$)

Table 2 Global hierarchical analysis of molecular variance (AMOVA) as a weighted average over all loci, for genetic clusters returned by STRUCTURE. (n = 5 for *P. nettingi*, n = 4 for *P. cinereus*)

Source of variation	Variance components	% of variation	P-value
P. nettingi			
Among clusters	0.38	14.21	< 0.001
Among locations within clusters	0.18	6.92	< 0.001
Within locations	2.12	78.87	< 0.001
P. cinereus			
Among clusters	0.42	12.37	< 0.001
Among locations within clusters	0.22	6.44	< 0.001
Within locations	2.78	81.23	< 0.001

(Fig. 2). A relatively higher level of mtDNA variation in *P. cinereus* is not surprising considering the substantially larger geographic range and the presumably larger effective population size of this species compared to *P. nettingi*. This difference in mtDNA variation also is reflected in the haplotype trees for the two species, with the *P. nettingi* tree having relatively little diversity compared to *P. cinereus* (Supplementary Information Fig. S1). Moreover, the greater haplotype diversity in *P. cinereus* is partitioned across sampling sites, such that most populations contain multiple haplotypes, a pattern found only in one population of *P. nettingi* (Fig. 3a, b). Further supporting these findings, *P. cinereus* exhibited substantially higher values of nucleotide diversity and number of nucleotide differences between haplotypes compared to *P. nettingi* based on the mtDNA data.

Our samples from *P. cinereus* came from the same habitat patches as those from *P. nettingi* and, as such, might also be expected to exhibit fairly low levels of genetic variation. In fact, in a similar comparative study, Page et al. (2020) actually found *P. cinereus* to have lower levels of genetic diversity than another small-ranged, Appalachian mountain-top plethodontid salamander, *P. hubrichti*. However, based on our analyses of the microsatellite data, the levels of genetic variation we observed for *P. cinereus* from our sampled patches are substantially higher than those observed for *P. nettingi* (Table 1), and similar to values reported from *P. cinereus* from fragmented forest patches elsewhere in the species' range, *i.e.,* Indiana (Jordan et al. 2009), Ohio (Wilk et al. 2020) and Canada (Noël et al. 2007; Noël and Lapointe 2010). These data suggest that local population sizes and between-patch dispersal in *P. cinereus* is often high enough to offset the loss of genetic variation due to genetic drift. In contrast, the lower levels of genetic variation we observed in *P. nettingi* (Table 1) suggest that the opposite is true for this species.

As was seen in the mtDNA, patterns of spatial genetic structure in the microsatellite data differed between species as well. While all pairwise values of F_{ST} were significant, we found that, on average, values were higher in *P. nettingi*, indicating greater levels of population differentiation among sampling sites (Supplementary Information Table S2).

Table 3 Bottleneck detection under the TPM model (with p-values) and allele frequency distribution

Locality	TPM		Mode shift	
	P. nettingi	*P. cinereus*	*P. nettingi*	*P. cinereus*
Stuart knob	0.406	0.679	L-shaped	L-shaped
McGowan	0.769	0.453[a]	L-shaped	**Shifted**[a]
Blackwater	**0.054**		L-shaped	
CVNWR	0.875	0.843	L-shaped	L-shaped
Timberline	**0.019**		**Shifted**	
Dolly sods	**0.004**	0.679	L-shaped	L-shaped
Spruce knob	0.321	0.472	**Shifted**	L-shaped
John's camp	0.406[a]		L-shaped[a]	
Guadineer	0.711[a]	0.769[a]	L-shaped[a]	**Shifted**[a]
Snowshoe	0.922[a]	0.527	**Shifted**[a]	L-shaped
Genetic cluster	TPM		Mode shift	
	P. nettingi	*P. cinereus*	*P. nettingi*	*P. cinereus*
A1	0.875		L-shaped	
A2	0.188		L-shaped	
B1	0.527		L-shaped	
B2	**0.004**[b]		L-shaped	
B3	0.156		L-shaped	
A		0.629		L-shaped
B		0.875		L-shaped
C1		0.472		L-shaped
C2		0.527		L-shaped

Bolded values are significant at $p < 0.05$

Parameters for the TPM include 95% stepwise mutations and a 12% variance on multi-step mutations. "L-shaped" distributions under the mode-shift test indicate a failure to detect a bottleneck. All results are based on 50,000 permutations

[a]Localities with < 8 individuals

[b]This cluster only contains one locality (Dolly Sods)

Isolation-by-distance was also apparent in *P. nettingi*, with populations in close geographic proximity having higher genetic similarity (Fig. 4). Conversely, *P. cinereus* populations separated by 20 km had levels of genetic differentiation similar to those separated by 100 km. *P. cinereus* is thought to exhibit less sensitivity to habitat fragmentation than many other forest-dwelling salamander species (Jordan et al. 2009). They have been found at high densities in heavily disturbed habitats (Apodaca et al. 2012) and are able to travel through inhospitable environments, such as open fields (Marsh et al. 2004).

On a broader scale, the east–west division seen in the geographic distribution of haplotypes (Fig. 3a, b) is mirrored in the distribution of genetic clusters from STRUCTURE (Fig. 3c, d), which coincides with the topography of the region. There are two ridges of high elevation forest which run roughly along the paths of the two main *P. nettingi* clusters and are separated by a relatively low elevation valley, which appears to limit gene flow in both species. These results contrast with those from another mountain-top plethodontid salamander, *P. shenandoah*, a species found on only three disjunct mountaintops in the Appalachians; Mulder et al. (2019) concluded that dispersal across north-facing slopes at mid-elevation levels likely played an important role in shaping genetic structure among the extant populations.

Within each forest ridge, the pattern of population structuring showed interesting differences between species. While more genetic clusters were identified in *P. cinereus* (K = 3 vs. K = 2), they spanned multiple localities which could result from high elevation habitat corridors or from individuals moving into mid-elevations to travel between populations. *P. nettingi* showed greater substructuring within clusters, suggesting that dispersal even within forest habitats is restricted. Although *P. nettingi* have been found to survive in somewhat warmer temperatures (and therefore lower elevations) in the absence of interspecific competition (Pauley and Clovis 1980), contemporary populations have relatively low genetic diversity, high population genetic differentiation,

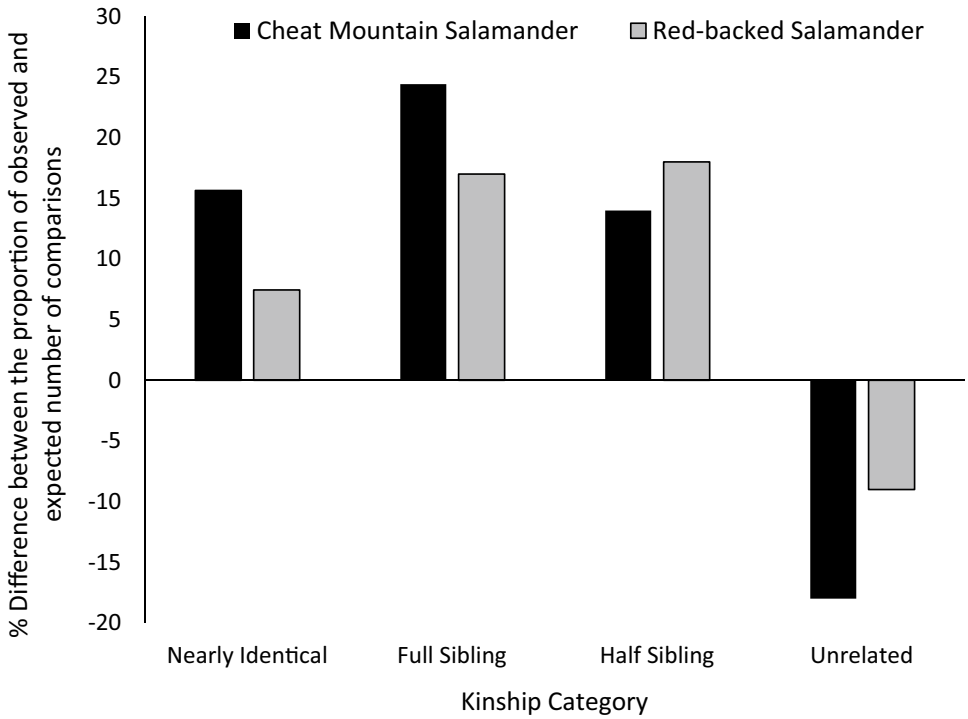

Fig. 5 Proportion of kinship observed for each species that is more than expected levels due to chance in each of four kinship categories

and evidence of having experienced both older and more recent population bottlenecks (Table 3).

Further evidence of limited contemporary gene flow among populations of *P. nettingi* is reflected in the significant kin groupings seen in this species (Fig. 5). Kinship, especially when coupled with strong breeding site philopatry, has been shown to be an important driver of population genetic structure in other amphibians with fragmented geographic distributions (Arbogast et al. in press). Although kin groupings were also seen in *P. cinereus*, the distribution of these groupings was different than that observed in *P. nettingi*; for example, whereas we found that *P. nettingi* sites were comprised of many more full-siblings and fewer unrelated individual than would be expected by chance, *P. cinereus* tended to have more half-siblings. Importantly, *P. nettingi* sites also had a greater proportion of "nearly identical individuals", which is indicative of high genetic similarity (Fig. 5). Overall, our data strongly suggest that *P. nettingi* has more restricted gene flow and lower connectivity than *P. cinereus* across the geographic area of our study. This is likely due to the stricter habitat requirements of *P. nettingi* and the inhospitable environments currently found at intervening lower elevations.

Management implications and conclusion

Our study indicates that *P. nettingi* could benefit from management practices aimed at enhancing genetic diversity and management strategies for other mountain-top plethodontid salamanders are already in place. For *P. hubrichti* and *P. punctatus,* "species of concern" at the federal level, this includes protection of their high-elevation habitat from logging and the development of wind energy (USFS 2014). Moreover, logging in secondary habitat of *P. hubrichti* must occur outside of periods of surface activity, and woody debris is left at those sites where logging does take place (Bayer et al. 2012). For *P. nettingi* in particular, accelerating red spruce forest recovery in the central Appalachians could potentially lead to enhanced or increased habitat. Predictive models indicate that opportunities for restoration may exist in areas where red spruce currently is sparse or absent, but where environmental variables such as elevation, precipitation and growing days are suitable (Nowacki and Wendt 2009). For instance, in areas where red spruce occurs in scattered stands, expanding and reconnecting these small, disjunct patches may prove beneficial, improving habitat conditions for *P. nettingi* and other forest obligate species. Moreover, simulations have shown that, in areas where red spruce was initially abundant, releasing red spruce understory trees from competing overstory hardwoods should roughly double the basal area of red spruce and yield a mixed spruce–hardwood stand in as little as 10 years (Rentch et al. 2007). Future work should focus on identifying specific attributes of the landscape that may act as dispersal corridors for *P. nettingi*. For example, site-level analyses indicated that, in addition to areas with high densities of red spruce, the probability of *P. nettingi* occurrence at a fine spatial scale increased in areas with shallower depth to rock,

areas proximal to rocky outcrops but distal to seeps, and high elevation sites with sandstone geology (Schuler et al. 2002; Dillard et al 2008a, b).

In addition to red spruce habitat management practices, approaches geared at increasing genetic diversity within the remaining small, isolated populations of *P. nettingi* could have important benefits. Although conservation biologists traditionally have been hesitant to perform translocations (Frankham et al. 2017), a growing cadre of scientists (e.g. Frankham et al. 2017; Ralls et al. 2018, 2020) have recently advocated for a more proactive approach. These authors argue that the potential benefits of translocations, such as genetic rescue and decreased inbreeding depression, far outweigh the potential negative consequences of genetic mixing, such as outbreeding depression. The Cheat Mountain salamander shows genetic characteristics that translocations could address as part of a genetic management plan and parameters such as those collected in this study are essential in developing such a plan. Furthermore, a comprehensive genetic management plan for this species will also need to incorporate the predicted impacts of anthropogenically driven climate change (Frankham et al. 2017; Gaitán-Espitia and Hobday 2021) and establish monitoring protocols and targets for assessing whether proposed management actions for the federally threatened Cheat Mountain salamander produce the desired genetic benefits (Van Rossum and Hardy 2020).

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Data availability All mtDNA sequences have been deposited in GenBank (accession numbers KX061194-KX061293 for *P. cinereus* and KX061294-KX061401 for *P. nettingi*). Microsatellite data are available from the authors upon reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Informed consent Consent granted.

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