RESEARCH ARTICLE

Effects of roads on patterns of genetic differentiation in red-backed salamanders, *Plethodon cinereus*

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Abstract Roads can fragment animal populations by reducing gene flow, which can lead to drift and the loss of genetic diversity. One of the principle signatures of reduced gene flow is increased genetic differentiation in isolated populations, and evidence that roads contribute to such differentiation has been reported for several species. We used microsatellites to examine whether six roads led to increased genetic differentiation in red-backed salamanders (*Plethodon cinereus*). These six roads included one divided interstate highway, one undivided four-lane highway, and four secondary roads. We found that the genetic distance between plots that were bisected by the interstate highway was significantly greater than the genetic distance between equidistant plots on the same side of the highway. However, for the five smaller roads, plots across the road were no more genetically distinct than were plots on the same side of the road. Bayesian clustering methods also supported both of these findings. The optimal clustering of plots for the interstate highway consisted of two clusters that corresponded to the two sides of highway. For the other five sites, the optimal grouping consisted of a single cluster containing all of the plots. Our findings suggest that gene flow across very large roads is rare and that bisected red-backed salamander populations are likely to diverge from one another. For smaller roads, our results imply that the indirect effects of roads on genetic population structure are probably less of a pressing concern for terrestrial salamanders than are the direct effects of mortality and habitat alteration.

Keywords Fragmentation · Amphibian · Highways · Dispersal · Gene flow

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Introduction

Roads can have a variety of negative effects on animal populations. For one, collisions with vehicles can be a major source of direct mortality for many species (see Trombulak and Frissell 2000 for review). Estimates of roadkill mortality for slow-moving taxa like amphibians range as high as 10% of the population per year (Hels and Buchwald 2001). For turtles, road mortality has been implicated in population declines and skewed sex ratios across species and the entire United States (Gibbs and Shriver 2002; Gibbs and Steen 2005). In addition, roads may contribute to the fragmentation of critical habitats. Some species may be resistant to crossing roads (Oxley et al. 1974; Swihart and Slade 1984; Dyer et al. 2002), or may avoid roads entirely (Merriam et al. 1989), leading to reduced dispersal across roads. This reduced dispersal can have important consequences for population dynamics. Reduced dispersal may disrupt metapopulation dynamics and prevent recolonization of suitable but unoccupied

habitat (Hanski 1991; Sjögren-Gulve and Ray 1996). But even in the absence of classical metapopulation dynamics (*sensu* Hanski 1999), reduced dispersal could eliminate the potential for rescue effects (Hels and Nachman 2002; Jaeger and Fahrig 2004), meaning that populations headed towards local extinction would be unlikely to be augmented by immigrants from other areas.

Reduced dispersal due to roads may also have negative consequences for the genetic structure of populations. If roads reduce or eliminate gene flow among populations, genetic diversity may be lost and populations isolated by roads may become increasingly divergent from one another (e.g., Gerlach and Musolf 2000; Epps et al. 2005). Although roads generally constitute recent barriers to dispersal, use of highly variable genetic markers has allowed researchers to detect the effects of roads on patterns of genetic structure in animal populations (Epps et al. 2005; Riley et al. 2006).

Previous studies of the effects of roads on genetic differentiation have generally taken one of two approaches. The first approach is to assess genetic differentiation between pairs of populations over large geographic areas and attempt to correlate these genetic distances with various measures of geographic isolation. Studies of common frogs (*Rana temporaria*: Reh and Seitz 1990; Hitchings and Beebee 1997), ground beetles (*Carobus violaceous* and *Abax parallelepipedus*: Keller and Largiadèr 2003; Keller et al. 2004), and desert bighorn sheep (*Ovis canadensis nelsoni*: Epps et al. 2005) all found that measures of isolation that included road effects were better predictors of genetic distance than were measures of isolation that included only geographic distance. The second approach to investigating the effects of roads on genetic differentiation is to trap animals from both sides of individual roads and then use either genetic distance measures or assignment methods to compare individuals or groups on the same side of each road to individuals or groups on the opposite side of the road. This latter method has the advantage of permitting one to make inferences about the effects of individual roads, and it has recently been used to demonstrate the effects of highways on gene flow in bank voles (*Clethrionomys glareolus*: Gerlach and Musolf 2000), bobcats (*Lynx rufus*), and coyotes (*Canis latrans*: Riley et al. 2006).

We used this latter approach to examine the effects of individual roads on genetic differentiation in red-backed salamanders, *Plethodon cinereus*, in the Appalachian mountains of Virginia and West Virginia, USA. Red-backed salamanders provide an interesting comparison to previous studies of the effects of roads on genetic differentiation because they appear to disperse less than most animals previously studied (Gillette 2003; Cabe et al. 2007), which should contribute to increased genetic differentiation.

However, red-backed salamanders also have very high population densities (>2/m^2 in some populations: Jaeger 1980a; Mathis 1991), and this should tend to reduce the effects of genetic drift in isolated populations. Additionally, the barrier effects of smaller roads on red-backed salamander dispersal have been studied with pitfall trapping and mark-recapture techniques (Gibbs 1998; DeMaynadier and Hunter 2000; Marsh et al. 2005), so genetic patterns can be evaluated in light of dispersal data. From a conservation perspective, red-backed salamanders are an important study organism because they are behaviorally and physiologically similar to a number of threatened and endangered salamanders (Highton and Larson 1979) whose small ranges and low densities would make analogous studies logistically difficult or unethical to carry out.

We collected genetic samples from red-backed salamanders in plots that were equidistant from one another and that were either separated by a road or by continuous forest. We did this at six roads, including one interstate highway, one four-lane highway, and four two-lane roads that varied in width and age. We amplified six microsatellite loci from each sample using markers that have previously been effective for elucidating patterns of genetic differentiation in red-backed salamanders (Cabe et al. 2007). For each of the six sites, we used hypothesis tests based on genetic distance measures and Bayesian clustering methods to determine whether individual roads contribute to genetic differentiation in red-backed salamander populations. We also examined the possible effects of road size and road age on the magnitude of any such differentiation.

Methods

Study species

Red-backed salamanders inhabit moist, deciduous forest from North Carolina north to Nova Scotia and west to Minnesota (Petranka 1998). They are most commonly found under rocks and logs on the forest floor, though the majority are usually underground at any given time (Test and Bingham 1948; Taub 1961). Red-backed salamanders have a completely terrestrial life-history: adults lay eggs underneath rocks and logs or in the leaf litter, and hatchlings emerge as terrestrial juveniles. Red-backed salamanders reach sexual maturity at 2–3 years of age (Petranka 1998) though they probably have a life-span at least twice this long. They can reach very high densities in mature forests and can be major contributors to energy and nutrient flow in these ecosystems (Burton and Likens 1975; Davic and Welsh 2004).

Recent studies have begun to shed light on dispersal and gene flow in red-backed salamanders. Adult salamanders have small home ranges, on the order of 10–25 m^2 (Kleeberger and Werner 1982). While juveniles may engage in some longer-distance dispersal, these movements appear to be on the order of tens of meters rather than hundreds of meters (Marsh et al. 2004). Both mark-recapture data (Gillette 2003) and analysis of genetic population structure (Cabe et al. 2007) suggest similarly limited dispersal. Populations of red-backed salamanders in Virginia show small, but detectable differentiation over scales of only a few hundred meters, and differentiation is closely related to geographic distance through continuous forest (Cabe et al 2007). With respect to roads, both pitfall trapping (Gibbs 1998; deMaynadier and Hunter 2000) and displacement experiments (Marsh et al. 2005) suggest that wide logging roads and narrow paved roads are partial barriers to salamander movement, with roads decreasing movement by 25–75%.

Study sites and sampling design

We collected DNA samples from salamanders on both sides of six paved roads in Virginia and West Virginia, USA. These roads included four, two-lane state roads (VA–613, VA–658, WV–17, the Blue Ridge Parkway) one four-lane federal road (US–460), and one divided, four-lane interstate highway (I–64). The width of the paved surface of the roads varied from 5 m to 42 m (Table 1) and the total width of the road and the adjacent gravel and grassy verge ranged from 13 m to 104 m (Table 1). Roads were constructed between 35 and >150 years ago (Table 1).

All sampling sites were areas where roads bisected mature forest inhabited by red-backed salamanders. We avoided any sites with streams that paralleled the road or that bisected any pair of plots, as streams appear to be partial barriers to red-backed salamander dispersal and gene flow (Marsh et al. 2007). We also avoided sites with any obvious signs of disturbance (e.g., burning, timber removal, etc.) that could confound analysis of the effects of roads on genetic differentiation. To the extent possible, we attempted to locate sites on flat terrain, though natural hillsides alone do not appear to be strong barriers to salamander dispersal (Marsh et al. 2005). Four sites were

located within National Forest areas, one was within Shenandoah National Park, and one was on private land.

Each sampling grid consisted of four, 50 m × 50 m plots arranged at the vertices of a 200 m-long square that was bisected by each road (Fig. 1). Within each plot, we attempted to collect genetic samples from 48 red-backed salamanders. In several cases, we were not able to achieve the desired 48 samples, and in other cases we collected somewhat more than this number (Table 2). In three cases (VA-658, WV-17, and I-64), we failed to find any salamanders in one of the four plots at a site. In these cases, we simply analyzed the effects of roads by comparing the two plots bisected by the road to the two equidistant plots on the same side of the road. All sampling took place between July 2002 and October 2005 and collection at each individual site was normally carried out over several weeks within this period. No collections required longer than five months and no samples spanned multiple reproductive periods for red-backed salamanders.

Sample collection and genetic analysis

We captured salamanders from under rocks and logs or within the leaf litter. From each captured salamander we removed approximately 1 cm from the tip of the tail for genetic analysis. We then re-released salamanders at the site of capture. Red-backed salamanders commonly lose their tails in nature and re-grow them within several weeks (Jamison and Harris 1992). Thus, we did not remove tissue from any salamanders with incomplete tails, as this might lead to repeated sampling of individual salamanders.

DNA was extracted from tail tissue following the protocols detailed in Connors and Cabe (2003) and Cabe et al. (2007). Briefly, we placed tail tissue in a sterile 1.5 ml microtube containing collection buffer (10 mM Tris, 10 mM EDTA, pH 8), put these tubes on ice for transport, and then performed DNA extraction using Promega Wizard Genomic DNA isolation kits within 24 h. We ground tissue samples in 500 µl of Nuclei Lysis Solution, incubated at 65°C for 20–30 min, treated with RNase at 37°C for 25 min, treated with 170 µl of Protein Precipitation Solution, and then centrifuged these samples. The supernatant was decanted and the DNA was precipitated using isopropanol. The DNA pellet was then washed with 70%

Table 1 Size and history of the roads used in our study

Road	Paved width (m)	Total width (m)	Approximate age (yrs)	Notes
I-64	42	104	35	Total width includes median strip
US-460	28	47	>150	Widened to four lanes in 1968
WV-17	6	16	>80	
BRP	7	13	65	
VA-658	6	13	?	Road age unknown
VA-613	5	22	>100	

Listed ages > 100 years include road use for carriages and other pre-motorized vehicles
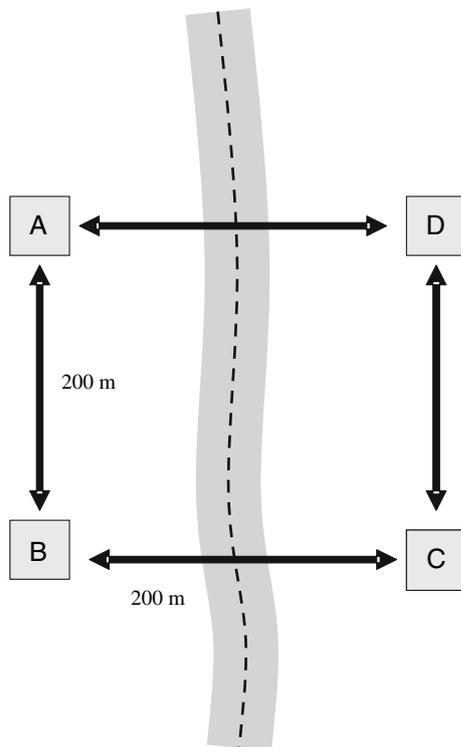
Fig. 1 Sampling design for each of the road sites. Approximately 48 salamanders were collected from each of the plots **A**, **B**, **C**, and **D**

ethanol, dried, and rehydrated with TE. DNA samples were stored in a freezer and dilutions of this stock (1:4 or 1:9) were used as templates for PCR.

We amplified six microsatellite loci (PcI16, PcLX16, PcLX23, PcJX06, PcFX08, and PcXD23) following Connors and Cabe (2003) and Cabe et al. (2007). We amplified an additional locus (PcPX07) for two sites but dropped it after finding evidence of linkage to PcXD23 at one of the sites. After verifying the success of our PCR via agarose gel electrophoresis, PCR products were shipped to the Advanced Genetics Analysis Center at The University of Minnesota where they were sized using ABI 377 or 3100 DNA sequencers and Genescan software. We determined genotypes in our laboratory using Genotyper software. We manually inspected each allele call, and binned alleles accordingly.

Data analysis

Summary statistics

We used Microsatellite Analyzer (MSA) to obtain observed and expected heterozygosities, allele counts, and size ranges (Dierenger and Schlotterer 2003). We then used GenePop 3.4 (Raymond and Rousset 1995) to test each

locus for Hardy–Weinberg equilibrium and for linkage disequilibrium. The probabilities from these tests were adjusted for an experiment-wise probability of 0.05 using the sequential Bonferroni correction (Weir 1990). For any loci still out of H–W equilibrium after the Bonferroni correction, we used Microchecker 2.2.3 (van Oosterhout et al. 2004) to check for evidence of null alleles, allelic dropouts, or mis-scoring. We then re-ran any relevant analyses dropping problematic loci or else correcting them for potential null alleles.

Testing for road effects

We used resampling methods (Manly 1997) to test the hypothesis that sub-populations on opposite sides of each road (i.e., A–D and B–C in Fig. 1) are more genetically distinct than equidistant subpopulations on the same side of each road (i.e., A–B and C–D). We previously used identical methods to test the effects of a stream on differentiation (Marsh et al. 2007). We based the resampling analysis on measures of the pairwise genetic distance for each pair of adjacent plots. To ensure that our results did not depend on the chosen distance metric, we used three different measures of pair-wise genetic distance: F_{ST} (Weir and Cockerham 1984), Nei's genetic distance (Nei 1972), and Reynolds' genetic distance (Reynolds et al. 1983). For hypothesis testing, our test statistic was the mean genetic distance between adjacent plots on opposite sites of the road minus the mean genetic distance between plots on the same side of the road (i.e., (A–D + B–C)/2 – (A–B + C–D)/2). Positive values of the test statistic indicate that across-the-road plots are more genetically distinct than same-side plots, negative values indicate that same-side plots are more distinct, and zero is expected under the null hypothesis that the road does not influence genetic differentiation. We did not include diagonal plots in these comparisons (i.e., A–C and B–D) as this would have introduced distance as a confounding factor.

For significance testing, we first calculated the value of the test statistic for the actual data. We then constructed 95% confidence intervals for this value by bootstrapping multilocus genotypes within plots 1,000 times (Marsh et al. 2007). Finally, we asked whether confidence intervals overlapped zero, as would be expected under the null hypothesis that the roads did not influence genetic differentiation. We repeated this analysis for each distance measure. Because these different distance measures represent highly non-independent checks for consistency rather than independent post-hoc comparisons, we used an alpha of 0.05 for each distance measure. We also note that our method of bootstrapping multilocus genotypes within plots is analogous to the determination of hierarchical F_{ST}'s by existing statistical

Table 2 Descriptive statistics for microsatellite loci at each of the six sites

SITE	LOCUS	N	Alleles	H_O	H_E	F_{ST}
I-64	Pcl16	127	4	0.02	0.08	0.0007
I-64	PcLX16	142	12	0.74	0.77	0.001
I-64	PcLX23	137	15	0.49	0.63	0.0751
I-64	PcJX06	121	6	0.23	0.52	0.0154
I-64	PcXD23	135	10	0.90	0.86	−0.0024
I-64	PcPX07	136	10	0.90	0.87	−0.0038
US-460	Pcl16	182	4	0.36	0.36	−0.0037
US-460	PcLX16	181	9	0.76	0.73	0.0015
US-460	PcLX23	181	8	0.44	0.45	0.0022
US-460	PcJX06	181	12	0.79	0.84	0.0054
US-460	PcFX08	157	12	0.76	0.75	−0.0008
US-460	PcXD23	178	14	0.71	0.74	0.0039
WV-17	Pcl16	169	4	0.34	0.41	0.0408
WV-17	PcLX16	178	6	0.47	0.50	0.0177
WV-17	PcLX23	177	10	0.63	0.64	0.0117
WV-17	PcJX06	174	9	0.60	0.58	0.0531
WV-17	PcFX08	173	14	0.74	0.79	0.0056
WV-17	PcXD23	162	11	0.64	0.77	0.0067
BRP	Pcl16	182	10	0.64	0.72	−0.0002
BRP	PcLX16	179	19	0.88	0.89	0.0023
BRP	PcLX23	182	14	0.88	0.87	−0.0001
BRP	PcJX06	187	15	0.91	0.89	−0.0014
BRP	PcFX08	175	23	0.88	0.92	−0.0011
BRP	PcXD23	171	28	0.81	0.87	0.0393
VA-658	Pcl16	82	2	0.03	0.17	−0.0176
VA-658	PcLX16	74	6	0.60	0.61	0.0069
VA-658	PcLX23	79	8	0.56	0.56	−0.0045
VA-658	PcJX06	78	13	0.89	0.87	−0.0059
VA-658	PcFX08	81	12	0.87	0.87	0.0043
VA-658	PcXD23	76	9	0.79	0.81	0.0121
VA-613	Pcl16	191	3	0.43	0.47	−0.0001
VA-613	PcLX16	191	6	0.65	0.68	−0.0023
VA-613	PcLX23	190	9	0.63	0.60	0.0002
VA-613	PcJX06	192	15	0.71	0.81	0.0091
VA-613	PcFX08	166	13	0.86	0.86	−0.001
VA-613	PcXD23	191	12	0.86	0.84	0.0009

Columns show number of samples (N), number of alleles, observed and expected heterozygosities, and F_{ST} across the site for each locus

packages such as GDA (Lewis and Zaykin 2001), but with comparisons restricted to plots that are equidistant to one another.

The approach described above tested for a road effect at each site separately. We also used a resampling approach to test for an overall effect of roads on genetic structure across sites. For this analysis, we asked whether the genetic distance between across-the-road plots was significantly larger than the genetic distance between same-side plots. To evaluate this hypothesis, we randomized whole plots within sites, rather than genotypes within plots. Because there were only three or four plots within each site and bootstrapping performs poorly at small sample sizes (Manly 1997), we shuffled plots without replacement rather than sampling them with replacement (i.e., permutation instead of boot-

strapping). For 1,000 iterations, we compared the t-value for the mean difference in genetic distance between cross-road plots and same-side plots to the value observed for the actual data. We used the proportion of t-values from the randomization that were greater than actual t-value as an estimate of the P-value for this hypothesis test. Finally, we used linear regressions to ask whether either road width or total width (road width plus width of roadside verges) were significant predictors of the degree of genetic differentiation at each site. For these regressions, we used the difference in F_{ST} values between adjacent cross-road plots and same-side plots as our index of differentiation. All resampling analyses were carried out in Matlab 7.0.4 and code is available from the senior author. Regression analyses were performed in S+ version 3.2.

To examine our data from another perspective, we used the program BAPS 4 (Bayesian Analysis of Population Structure; Corander et al. 2004; Corander and Marttinson 2006) to carry out further analyses of the effects of roads on genetic differentiation. BAPS uses stochastic optimization to cluster individuals or groups based on their genetic similarity and on deviations from Hardy–Weinberg equilibrium that could be attributed to population structure within a dataset (Corander et al. 2003). We used uniform priors for all analyses in BAPS to see if evidence of differentiation due to each road could be uncovered without any prior information about the geographical origin of sampled individuals. We first used BAPS to cluster individuals from each site into genetic units. We determined the optimal number of clusters (i.e., the number that minimized the deviations from H–W equilibrium), using the number of plots as the maximum number of clusters permitted for each site. However, because BAPS often tends to identify relatively large numbers of clusters when genetic differentiation is subtle (Latch et al. 2006), we also examined in detail the optimal two-population clustering for each site. For each two-population clustering, we determined the extent to which the most likely clustering corresponded with the two sides of the road. Specifically, we calculated the percentage of individuals for whom the assigned cluster was indicative of the side of the road from which they were collected. This percentage would have a minimum value of 50% if each genetic cluster contained an equal number of individuals collected on each side of the road, and it would have a maximum value of 100% if the two genetic clusters identified by BAPS corresponded perfectly with the two sides of the road. Because some clusters were larger than others and equal numbers of samples were not always collected from both sides of the road, the expected correspondence between genetic clusters and sides of the road due to chance was often somewhat higher than 50%. Thus, we used a chi-squared statistic to measure the deviation between the observed clustering and the expected clustering under the null hypothesis that the road had no effect on genetic differentiation.

Finally, we used BAPS to cluster whole plots (rather than individuals) within each of the sites. For these analyses, we asked whether the optimal clustering of plots corresponded with the two sides of the road. For example, if plots A and B clustered together and plots C and D clustered together (see Fig. 1), this would support the hypothesis that the road which subdivides these pairs is a barrier to gene flow. Alternatively, if the optimal grouping was A with D and B with C, or if all plots grouped together as a single cluster, then this would provide no support for a barrier effect of the road.

Results

Summary statistics

The mean number of alleles per locus at each site ranged from 4.5 for Pcl16 to 14.8 for PcFX08 (Table 2). Mean heterozygosity ranged from 30% (Pcl16) to 82% (PcFX08; Table 2). Of the 126 plot/locus combinations, 104 were in Hardy-Weinberg equilibrium at alpha = 0.05. Using a Bonferroni correction for the number of comparisons yielded 115 of 126 values passably in equilibrium. The nine disequilibrial values were spread among four of the six loci, which suggests that no particular locus was subject to systematic problems. Microchecker 2.2.3 (van Oosterhout et al. 2004) found evidence for null alleles for each of the 9 plots out of equilibrium (at PcPX07, Pcl16, and PcJX06). Only PcPX07 and PcXD23 at the I-64 site exhibited any evidence of linkage. To be conservative, we removed PcPX07 from the analyses of this site to ensure that all markers could be considered informative. Comparison of results between analyses with and without PcPX07 showed no meaningful differences in estimated parameters or confidence intervals. Differentiation among plots within sites was generally low to moderate Mean F_{ST} for adjacent plots within a site was 0.018 for I-64, 0.003 for US-460, 0.029 for WV-17, 0.007 for the Blue Ridge Parkway, -0.002 for VA-568, and 0.003 for VA-613.

Road effects

Our resampling tests provided no evidence for an effect of roads on gene flow at the five smallest roads. For these five sites, genetic distance measures between cross-road plots were nearly equal to distance measures between same-side plots and none of these differences approached statistical significance (Table 3). However, for the interstate highway (I-64), there was evidence for increased genetic differentiation across the road. F_{ST}, Nei's genetic distance, and Reynolds' distance all gave significantly higher values for the plots across the road as compared to the plots on the same side of the road (Table 3). When all six sites were considered together, there was no overall effect of roads on genetic differentiation ($t = 0.33$, $P = 0.24$). A linear regression of either road width ($\beta = 0.0007$, $R^2 = 0.66$, $P = 0.048$) or total width ($\beta = 0.0003$, $R^2 = 0.83$, $P = 0.012$) on genetic differentiation (as indicated by F_{ST}) was significant, largely reflecting the leverage of the moderate differentiation and relatively large width at the I-64 site.

Results from the Bayesian clustering analyses were quite similar to the results from the resampling analyses. For all six sites, the optimal number of genetic clusters was equal to the total number of plots, probably as a result of

fine-scale population structure. However, when we fixed the maximum number of genetic clusters at two, the two clusters at the I-64 site had a 66% correspondence with the two sides of the road, and this association was significantly greater than would be expected by chance ($\chi^2 = 14.4$, df = 1, $P < 0.01$; Table 4). For all other sites, the correspondence between the most likely pair of genetic clusters and the two sides of the road was less than 60% and these correspondences were all within the range that would be expected by chance ($\chi^2 < 3$, $P > 0.10$ in all cases; Table 4). When whole plots were clustered within sites, the optimal grouping for I-64 was two clusters that corresponded with the two sides of the road, and the posterior probability for this clustering was 0.98. The second most likely clustering ($P = 0.02$) was for a single cluster containing all of the plots. For the other five sites, the optimal grouping of plots was a single cluster containing all of the plots, with posterior probabilities approaching one in every case.

Because we found evidence for null alleles at the I-64 site, and because null alleles can potentially increase the number of population clusters identified (Falush et al. 2003), we explored whether the apparent differentiation at the I-64 site was robust to the presence of null alleles. The two most problematic loci at the I-64 site (based on homozygote excess) were Pcl16 and PcJX06. When we deleted Pcl16 from the genetic distance (F_{ST}) analysis, the genetic distance across the road increased by 1% relative to the distance on the same side of the road. When we deleted PcJX06 from this analysis, the genetic distance across the road increased by 29%. We also attempted to correct the I-64 allele frequencies for possible null alleles instead of just deleting problematic loci. When we substituted in the corrected allele frequencies generated by Microchecker 2.2 (van Oosterhout et al. 2004), the genetic distance across the road increased by 13% relative to the genetic distance on the same side of the road. With respect to the clustering analysis, BAPS still grouped plots according to side of the highway with either of the two problematic loci removed. With Pcl16 removed, the posterior probability for this

grouping was 0.98 and with PcJX06 removed the posterior probability was 1.00. This grouping also existed when the dataset was corrected for null alleles, again with a posterior probability of 1.00. Thus, problems with null alleles do not appear to be responsible for the apparent effects of the road at the I-64 site. In all cases, attempts to delete or fix problematic loci resulted in a stronger road effect at this site.

Discussion

Our results suggest that a divided interstate highway has resulted in increased genetic divergence among red-backed salamander populations. At the same time, we found that several smaller roads do not appear to be affecting genetic population structure. These results were consistent across very different methods of genetic analysis .They were supported by both frequentist hypothesis tests based on patterns of genetic distance and by Bayesian clustering analyses using uniform priors for the grouping of individuals and plots.

These results can be interpreted in light of the various factors that should affect whether landscape barriers lead to genetic divergence. First, genetic divergence should be affected by the magnitude of gene flow between populations. Data from displacement experiments (Marsh et al. 2005) and pitfall trapping of dispersing animals (Gibbs 1998; deMaynadier and Hunter 2000) suggests that narrow, paved roads reduce red-backed salamander movement by approximately 25–75% but do not eliminate it entirely. Although dispersal does not always result in gene flow, particularly in territorial species (Whitlock and McCauley 1999; Riley et al. 2006), the lack of any detectable genetic divergence across smaller roads suggests that dispersal is probably sufficient to prevent divergence. The fact that genetic divergence across the interstate was detected implies that reductions in dispersal across this road are substantially greater than the 25–75% by which smaller

Table 3 Difference in genetic distance between plots across the road from one another and equidistant plots on the same side of the road

F_{ST}	Nei's	Reynolds	
Road	Difference (95% CI)	Difference (95% CI)	Difference (95% CI)
I-64	0.030 (0.003, 0.058)*	0.046 (0.009, 0.084)*	0.031 (0.006, 0.060)*
US-460	0.003 (−0.005, 0.010)	0.005 (−0.009, 0.019)	0.003 (−0.005, 0.010)
WV-17	0.007 (−0.015, 0.028)	0.011 (−0.029, 0.046)	−0.007 (−0.017, 0.028)
BRP	0.001 (−0.005, 0.008)	0.011 (−0.035, 0.061)	0.001 (−0.005, 0.008)
VA-658	−0.007 (−0.043, 0.018)	−0.016 (−0.076, 0.029)	−0.009 (−0.045, 0.013)
VA-613	0.001 (−0.007, 0.010)	0.004 (−0.016, 0.024)	0.001 (−0.007, 0.009)

Differences and confidence intervals are shown for genetic distance based on F_{ST}, Nei's distance, and Reynolds' distance. Differences that are significantly different from zero at an $\alpha = 0.05$ are marked with an asterisk

Table 4 Summary of Bayesian analysis of population structure. Plots refers to the number of plots at each site

SITE	Plots	Clusters (ind)	Correspondence with Road	χ^2	Clusters (plots)
I-64	3	3	0.66	14.34	2
US-460	4	4	0.52	0.23	1
WV-17	3	3	0.58	0.49	1
BRP	4	4	0.53	2.75	1
VA-658	3	3	0.60	0.76	1
VA- 613	4	4	0.53	0.34	1

Clusters (ind) are the optimal number of clusters of individuals selected by BAPS, which was always the maximum number that we permitted. Correspondence with Road is the proportion of individuals that were assigned to the side of the road from which they originated in the best two-cluster model. χ^2 is the deviation between the observed correspondence and the expected correspondence given cluster sizes and sample sizes for each plot. Clusters (plots) is the optimal number of clusters determined by BAPS when plots were clustered rather than individuals

roads reduce dispersal. Based on a regression of F_{ST} versus distance in continuous forest (Cabe et al. 2007), the observed divergence across I-64 ($F_{ST} = 0.033$) was equivalent to what would be expected over approximately 1,500 m of forest.

The second factor that should contribute to genetic divergence across landscape barriers is time. Establishing the age of roads is somewhat complicated. Many roads in this region existed as horse paths, wagon routes, or dirt logging roads before they were paved, and some roads were widened many years after they were first paved. That said, the age of the roads in our study varied widely (Table 1). US-460 and VA-613 appear to have existed in some form for more than 100 years (and perhaps 200 years), whereas the Blue Ridge Parkway and I-64 were the products of fairly recent construction. It is interesting, then, that the only road that led to increased genetic divergence (I-64) was in fact one of the most recently constructed. This suggests that genetic divergence can develop rather quickly when barriers to gene flow are strong, as has been demonstrated in studies of the effects of roads on other taxa (Keller and Largiadèr 2003; Keller et al. 2004; Epps et al. 2005). However, it should also be noted that using the same genetic markers, we found evidence of genetic divergence in red-backed salamanders across a second-order stream (Marsh et al. 2007) and that in displacement experiments, this stream reduced dispersal by about the same amount as the smaller roads in the present study (Marsh et al. 2005). Thus, it is possible that populations of red-backed salamanders across smaller roads would eventually diverge if given sufficient time.

The third main factor in determining the rate of genetic divergence across a barrier is effective population size. Previous studies of the effects of roads on genetic population structure have generally focused on species that maintain small effective population sizes. For example, a recent study of the effects of a highway on gene flow in carnivores (Riley et al. 2006) estimated an effective population size of 200 for bobcats (*Lynx rufus*) and 75 for

coyotes (*Canis latrans*). Ground beetles, *Carobus violaceous*, which were shown to have differentiated across roads in Switzerland (Keller and Largiadèr 2003), had densities of less 100 individuals per hectare (Keller et al. 2004). Desert bighorn sheep (*Ovis canadensis nelsoni*), which show reduced genetic diversity in populations isolated by roads, have similarly low population densities (Epps et al. 2005). Compared to each of these species, the effective population sizes of red-backed salamanders are quite large. Red-backed salamanders may reach densities above 2 per m^2 (Mathis 1991) over broad areas of forest. At this density, a genetic neighborhood with a 50 m radius would contain over 15,000 salamanders. Several of our sites (Blue Ridge Parkway and VA-613) may well have had red-backed salamander densities approaching 2 per m^2. Others (VA-460, VA-658, and I-64) appeared to have lower densities, and red-backed salamanders were sometimes difficult to find at these sites. The I-64 site may have had a particularly low effective population size because the back end of plots C and D was bordered by pasture. A reduced effective population size should allow drift to occur more quickly in the absence of sufficient gene flow between populations. Thus, the lack of dispersal across the interstate highway combined with somewhat lower population sizes may explain why we were able to detect divergence at this site in spite of the relative newness of the barrier.

There were several important limitations to our study. The first limitation was that with our sampling strategy any barrier to gene flow that ran parallel to the road would appear to be an effect of the road itself. Although we were careful to eliminate sites that had streams, ditches, or railroad tracks running parallel to the road, we cannot rule out the possibility that some other feature of the landscape may have caused the apparent barrier effect at the I-64 site. Narrow grassy strips, as occur in median strips on the verges of highways, do not appear to be barriers to red-backed salamander movement (Marsh et al. 2004). However, undetected features of the landscape cannot be

ruled out as barriers to gene flow. The second limitation of our study was that, as previously mentioned, red-backed salamanders often have very large population sizes. These large population sizes may make genetic drift a fairly weak force, even in the absence of substantial gene flow. It should be noted, though, that we have previously been able to detect fine-scale genetic structure over as little as three hundred meters of continuous forest (Cabe et al. 2007) and across a second-order stream (Marsh et al. 2007). Given that both of these findings were in areas with very dense red-backed salamander populations, it does not appear that large effective population sizes are by themselves able to prevent differentiation from occurring over small spatial scales. The third, and perhaps most important, limitation of our study is that it considered only six roads, and most of these were two-lane roads that were expected to be somewhat permeable to red-backed salamander dispersal (Marsh et al. 2005). Moreover, we were only able to include one interstate highway in the study. Therefore, although we did find significant genetic divergence across this highway, we cannot be certain of the generality of the result. We initially sought to include more large highways in our study but had difficulty finding sites with reasonable salamander densities that were adjacent to highways.

Nevertheless, our finding of significant divergence across the interstate was robust to both the statistical methods used and to some potential problems with homozygote excess at several of the loci. Furthermore, other evidence suggests that the high divergence across the interstate highway was not simply due to chance. The observed genetic distance between plots across the interstate from one another ($F_{ST} = 0.033$) was greater than any other F_{ST} that we have recorded for pairs of stream-free plots separated by 200 m (out of 28 pairs total in this study, in Cabe et al. 2007, and in Marsh et al. 2007). Finally, it makes intuitive sense that the one road for which we detected significant divergence was by far the largest road that we studied. Given this evidence, we tentatively suggest that large, divided highways should be expected to contribute to genetic divergence among red-backed salamander populations, but that no such divergence is likely to occur across secondary roads, at least over the time scales considered in this study.

Red-backed salamanders themselves are abundant and widely distributed and are not generally considered to be a species of conservation concern. However, red-backed salamanders are behaviorally and physiologically similar to a number of other terrestrial salamanders that have small ranges, and that may be declining or endangered. These species include the Shenandoah salamander, *Plethodon shenandoah*, a federally-endangered species which is restricted to three populations close to a major two-lane road (Highton and Larson 1979; Jaeger 1980b), the Peaks

of Otter salamander, *Plethodon hubrictii*, which lives only along a 20 km stretch of ridge that is bisected by the Blue Ridge Parkway (Highton and Larson 1979; Kramer et al. 1993), and the Cheat Mountain Salamander, a federally threatened species with a small range that includes numerous developed roads (Highton and Larson 1979; Pauley 1981). Obviously, there are caveats in drawing conclusions about the conservation of one species based on data from another (see Caro and O'Doherty 1999 for review). However, in the absence of detailed genetic studies of these threatened terrestrial salamanders, data from red-backed salamanders are probably the best surrogate available. Our data suggest that, from a conservation perspective, the most detrimental impact of small roads on terrestrial salamanders is probably *not* the creation of genetically isolated populations. Instead, the direct effects of these roads on population density (Marsh and Beckman 2004), on dispersal (deMaynadier and Hunter 2000; Marsh et al. 2005), and on direct mortality (Hels and Buchwald 2001; Mazerrolle 2004) are likely to be of greater conservation concern. On the other hand, our results also suggest that future construction of large highways, or widening of existing roads, should be considered a conservation issue for terrestrial salamanders, since it appears that these large roads may cause populations become genetically isolated from one another.

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