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Effects of forest fragmentation by secondary roads on genetic differentiation in northern red-backed salamanders (*Plethodon cinereus*)

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ABSTRACT

Roads can fragment animal populations, which can lead to decreased gene flow between populations. This causes increased genetic differentiation and decreased genetic diversity between and among populations. These affects are well documented in studies focusing upon the effects of urbanization on salamander populations, however, little is known about the effects of rural roads on fragmented populations of salamanders. We used microsatellites to examine the effects forest road fragmentation has on genetic diversity and differentiation in populations of northern red-backed salamanders. We sampled populations on both sides of two single-lane forest roads. Initial findings confirm successful PCR amplifications at the PcLX16 and PcI16 loci. Qualitative findings also show no observable heterozygosity. Genetic analyzer did not produce any usable data. Due to the lack of quantitative data, no conclusions can be made about the effects of forest road fragmentation on genetic differentiation.

INTRODUCTION

As human populations continue to grow, landscapes that were once contiguous are now increasingly fragmented. Roads, in particular, cause increased habitat fragmentation and can often create dispersal barriers between isolated populations (Gibbs 1998b, Gibbs 1998c, Kolozsvary and Swihart 1999, Marsh and Beckman 2004, Marsh et al. 2005). This can reduce gene flow and increase genetic differentiation (Gibbs 1998a, Cabe et al 2007, Marsh et al. 2008, Noel and Lapointe 2010). Roads and their effects on genetic differentiation and species dispersal have been studied heavily in urbanized areas (e.g., Noel et al. 2006). However, effects of small-scale landscape manipulations in predominantly rural areas remain largely unknown.

Amphibians respond negatively to habitat fragmentation caused by roads. Roads act as movement barriers to amphibians, inhibiting migration between fragmented populations. Road margins also produce an edge effect that exposes migrating amphibians to dessication, increasing mortality rate. This increase in mortality can also be attributed to road traffic as amphibians attempt to migrate across (Fahrig et al. 1995).

Habitat fragmentation also causes decreases in salamander population sizes, which can cause increased genetic drift, in turn increasing genetic differentiation (Noel and Lapointe 2010). This genetic differentiation can then negatively impact the survivorship of isolated populations (Balkenhol and Waits 2009) and can cause an increase in the number of recessive, detrimental alleles that would not normally be expressed in heterozygous states (Holderegger and Di Guilio 2010), compromising the genetic integrity of these isolated subpopulations of salamanders.

Plethodontid salamanders are susceptible to reductions in dispersal due to road-related fragmentation (Larson et al. 1984). Many studies (e.g., Marsh et al 2004) have concluded that forest fragmentation decreases salamander abundance and dispersal. Other studies (e.g., Noel et

al. 2006) have shown that major roads bisecting forested habitats have a substantial effect on genetic differentiation in salamanders. However, little is known about the possible effects that forest roads may have on genetic structure in fragmented populations.

The purpose of our study was to examine effects of habitat fragmentation by forest roads on genetic variation of northern red-backed salamander (*Plethodon cinereus*) populations. We chose to test the hypothesis that small forest roads will increase genetic differentiation and decrease genetic variation between fragmented populations of northern red-backed salamanders. We tested the effects of forest fragmentation on gene flow by using microsatellite markers (Connors and Cabe 2003) to compare genetic differentiation between populations separated by forest roads. We predicted that forest road fragmentation will decrease gene flow between populations of northern red-backed salamanders. Our results will help to provide an understanding of how forest roads affect genetic flow and thus migration of northern red-backed salamanders between isolated populations.

METHODS

Study species

We chose to study northern red-backed salamanders found in forested areas fragmented by seasonal forest roads in Kalkaska County, Michigan. Northern red-backed salamanders inhabit mature forest segments (Harding 1997) in areas with moist soil, under downed course woody debris (Quinn et al. 1999, McKenney et al. 2006). In areas where this species is found, it is considered to be the most common forest vertebrate with an effective population size of 500 to 9,000 salamanders per hectare (Harding 1997).This species, like other plethodontid salamanders, does not disperse across areas exposed to dryness and heat (Larson et al. 1984) and is vulnerable to desiccation

Study Area

We selected two road segments in Kalkaska County, MI (Site 1: 44.775937^0N, -84.923192^0 W; Site 2: 44.800253^0N, -84.934643^0W). Each segment is 100 m in length. Both roads are one lane, sand and gravel roads. Road segments were similar in surrounding habitat, comprised of northern hardwood forests (sugar maple (*Acer saccharum*), American beech (*Fagus grandifolia*), and yellow birch (*Betula alleghaniensis*)). Sites differ with respect to forest floor cover and surrounding features. Blackberry (*Rubus sp.*) is the predominant forest floor cover at site one while site two is predominantly bracken fern (*Pteridium aquifolium*). Soil composition is similar between the two sites. Both sites can be described as having sandy soil with a top layer of organic topsoil. Site two has two seasonal ponds while site one has no standing water. These seasonal ponds should have no effect on the distribution and abundance of redback salamanders since they are exclusively terrestrial.

Field Sampling Methods

We sampled sites during June and July 2011. We collected 24 northern red-backed salamanders at each site by randomly flipping over downed course woody debris within a 1 ha plot on each side of the road. Distance from the road margin was recorded for each salamander categorically (<20m; >20m). We collected tissue samples by clipping 1 cm of the distal end of the tail aseptically, using a flame sterilized set of surgical scissors (HACC 2004). We transported tails from the field site using an icebox and placed them in a storage freezer upon return. Each site was divided equally into 2 subpopulations from each side of the transecting road (N=12 for each subpopulation).

Molecular analyses

We extracted DNA using QIAGEN DNeasy tissue extraction protocol (QIAGEN, 2004), and genotyped all samples at four dinucleotide microsatellite loci (PcLX16, PcI16, PcLX23, and PcJX24) designed for northern red-backed salamanders (Connors and Cabe 2003). We then fluorescently labeled each forward primer by adding a set of tagged, universal primers to our PCR reaction (VIC, 6-fam, and NED).

We carried out PCR amplifications in 18.25 μl of sterile water, 3.0 μl of 10x reaction buffer with MgCl, 2 μl dNTPs, 0.1 μl of forward primer, 0.2 μl of reverse primer, 0.4 μl of fluorescent tag, 0.2 μl of Taq polymerase, and 1 μl of DNA sample, and performed touchdown procedures for each of our PCR amplifications (Wcisel personal comm.). Reaction cycling conditions were as follows: initial denaturation at 94°C for 2 min, touchdown from initial annealing temperature (10 cycles decreasing 1 ^{o}C each time (94 ^{o}C for 30s, starting annealing temperature for 45s, 72 ^{o}C 30s)), 30x (94°C for 30s, final annealing temperature for 45s, and 72°C for 30s), and a final extension of 72°C for 5 min for all loci (Wcisel personal comm., Connors and Cabe 2003). Initial annealing temperatures were as follows: PcLX16 - 65 ^{o}C; PcJX24 and PcLX23 - 60 ^{o}C; PcI16 - 55 ^{o}C).

PCR amplifications were run on an agerose gel to test for success and heterozygosity. Gels consisted of 0.7 g agerose, 100 ml water, and 10 μl red stain. We combined 1.0 μl of glycerol and 4.0 μl of DNA, and then loaded them into each gel well. We ran gel electrophoresis for six minutes. We observed gels under a UV light to test for success and observable heterozygosity.

We ran all successful PCR amplifications on an Applied Biosystems Inc. genetic analyzer. Results were visualized using Peak Scanner (Applied Biosystems Inc.) to provide quantitative data for analysis.

RESULTS

        We successfully tested the PCR amplifications at the PcLX16 and PcI16 loci; however

we were unsuccessful testing our other two loci (PcLX23, PcJX24). Agerose gels showed that

PCR amplification was successful for 37 out of 48 samples. Unsuccessful samples were

characterized by the lack of a strong product band or by excessive streaking within the lane (Fig.

1). Several individuals also showed multiple bands, which represent nonspecific products (Fig.

1). We saw no conclusive heterozygosity on the agerose gels for this primer set.

        We ran all PCR products for the two microsatellite loci on a genetic analyzer. We found

that 91 of our samples failed to produce any data (Fig. 2). Only five samples provided successful

results (Fig. 3), however these peaks were all weak, showed no patterns, and had incorporated

background noise.

DISCUSSION

        Our gels confirmed that we were indeed successful in amplifying DNA at the PcLX16

and PcI16 loci. Our gels did not suggest any observable heterozygosity, which seems to conflict

with our hypothesis. However, base-pair polymorphisms are often very minute, and may only be

several nucleotides in length (Evans personal comm.) meaning that the use of a genetic analyzer

is necessary to quantify our results. Without this quantifiable data, it is not possible to either

falsify or support our hypothesis based off our current findings.

        Continued research is necessary in order to reach a conclusion. Currently, we hope to

amplify our 48 samples at the 2 remaining loci and redo our analysis of the two aforementioned

loci. Once quantifiable data is obtained we intend to implement several statistical tests in order

to test for the degree of heterozygosity. We intend to calculate the fixation index (F_{st}), which

will be used to compare genetic diversity at our four microsatellite loci within our

subpopulations, as compared with the entire site (e.g. Marsh et al. 2008). We will also calculate the ratio of observed heterozygotes (H_o) to expected heterozygotes (H_e). Finally, we will compare each locus to the expected Hardy-Weinberg equilibrium in order to determine the degree and rate of genetic variation and evolution within populations (e.g., Marsh et al. 2008). This data will hopefully allow us to form significant conclusions over whether forest roads seem to impact genetic diversity and differentiation between fragmented subpopulations.

ACKNOWLEDGEMENTS

LITERATURE CITED

Balkenhol, N., and L.P. Waits. 2009. Molecular road ecology: exploring the

potential of genetics for investigating transportation impacts on wildlife. Molecular

Ecology 18:4151-4164.

Cabe, P.R., Page, R.B., Hanlon, T.J., Aldrich, M.E., Conors, L., and D.M. Marsh.

2007. Fine-scale population differentiation and gene flow in a terrestrial salamander

(*Plethodon cinereus*) living in continuous habitat. Heredity 98:53-60.

Connors, L.M., and P.R. Cabe. 2003. Isolation of dinucleotide microsatellite loci from

red-backed salamanders (*Plethodon cinereus*). Molecular Ecology Notes 3:131-133.

Fahrig, L., Pedlar, J.H., Pope, S.E., Taylor, P.D., and J.F. Wegner. 1995. Effect of road

traffic on amphibian density. Biological Conservation 73:177-182.

Gibbs, J.P. 1998a. Genetic structure of redback salamander Plethodon cinereus

populations in continuous and fragmented forests. Biological Conservation 86:77-81.

Gibbs, J.P. 1998b. Distribution of woodland amphibians along a forest fragmentation

gradient. Landscape Ecology 13:263-268.

Gibbs, J.P. 1998c. Amphibian movements in response to forest edges, roads, and

streambeds in southern New England. Journal of Wildlife Management 62:584-589.

Harding, J.H. 1997. Amphibians and Reptiles of the Great Lakes Region. The

University of Michigan Press:91-96.

Hobbs, R.J. 1992. The Role of Corridors in Conservation: Solution or Bandwagon?

Trends in Ecology and Evolution 7:389-392.

Herpetological Animal Care and Use Committee. 2004. Guidelines for the use of live

amphibians and reptiles in field and laboratory research. American Society of

Ichthyologists and Herpetologists.

Holderegger, R., and M. Di Guilio. 2010. The genetic effects of roads: A review of

empirical evidence. Basic and Applied Ecology 11:522-531.

Kolozsvary, M.B., and R.K. Swihart. 1999. Habitat fragmentation and the distribution

of amphibians: patch and landscape correlates in farmland. Canadian Journal of Zoology

77:1288-1299.

Larson, A., Wake, D.B., and K.P. Yanev. 1984. Measuring gene flow among

populations having high levels of genetic fragmentation. Genetics 106:293-308.

Marsh, D.M., and N.G. Beckman. 2004. Effects of forest roads on the abundance and

activity of terrestrial salamanders. Ecological Applications 14:1882-1891.

Marsh, D.M., Thakur, K.A., Bulka, K.C., and L.B. Clarke. 2004. Dispersal and

colonization through open fields by a terrestrial, woodland salamander. Ecology

85:3396-3405.

Marsh, D.M., Milam, G.S., Gorham, N.P., and N.G. Beckman. 2005. Forest roads as

partial barrier to terrestrial salamander movement. Conservation Biology 19:2004-2009.

Marsh, D.M. 2007. Edge effects of gated and ungated roads on terrestrial salamanders.

The Journal of Wildlife Management 71:389-394.

Marsh, D.M., Page, R.B., Hanlon, T.J., Corritone, R., Little, E.C., Seifert, D.E., and P.R.

Cabe. 2008. Effects of roads on patterns of genetic differentiation in red-backed

salamanders, *Plethodon cinereus*. Conservation Genetics 9:603-613.

McKenny, H.C., Keeton, W.S., and T.M. Donovan. 2006. Effects of structural

complexity enhancement on eastern red-backed salamander (*Plethodon cinereus*)

populations in northern hardwood forests. Forest Ecology and Management 230:186-196.

Noel, S., Ouellet, M., Galois, P., and F. Lapointe. 2006. Impact of urban fragmentation on the genetic structure of the eastern red-backed salamander. Conservation Genetics 2007:599-606.

Noel, S., and F. Lapointe. 2010. Urban conservation genetics: Study of a terrestrial salamander in the city. Biological Conservation 143:2823-2831.

QIAGEN. 2006. DNeasy Blood & Tissue Handbook. QIAGEN Group, Austin, Texas, USA.

Quinn, V.S., and B.M. Graves. 1999. Space use in response to conspecifics by the red-backed salamander (*Plethodon cinereus,* Plethodontidae, Caudata). Ethology 105:993-1002.

FIGURE LEGEND

Figure 1: Representative agerose gel of samples amplified at the PcLX16 locus. Strong, single bands represent successful PCR amplifications. Lanes with no banding or strong streaking represent unsuccessful amplifications. Lanes with multiple bands represent the product, along with several nonspecific products.
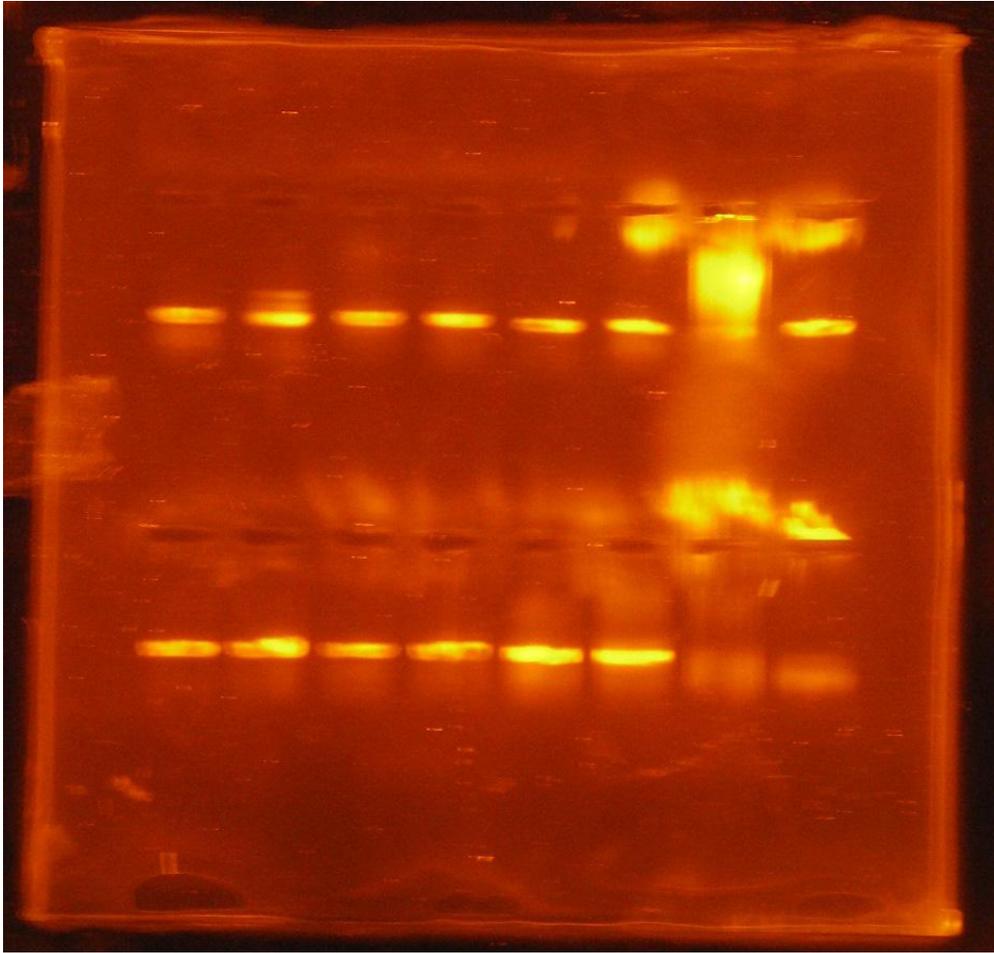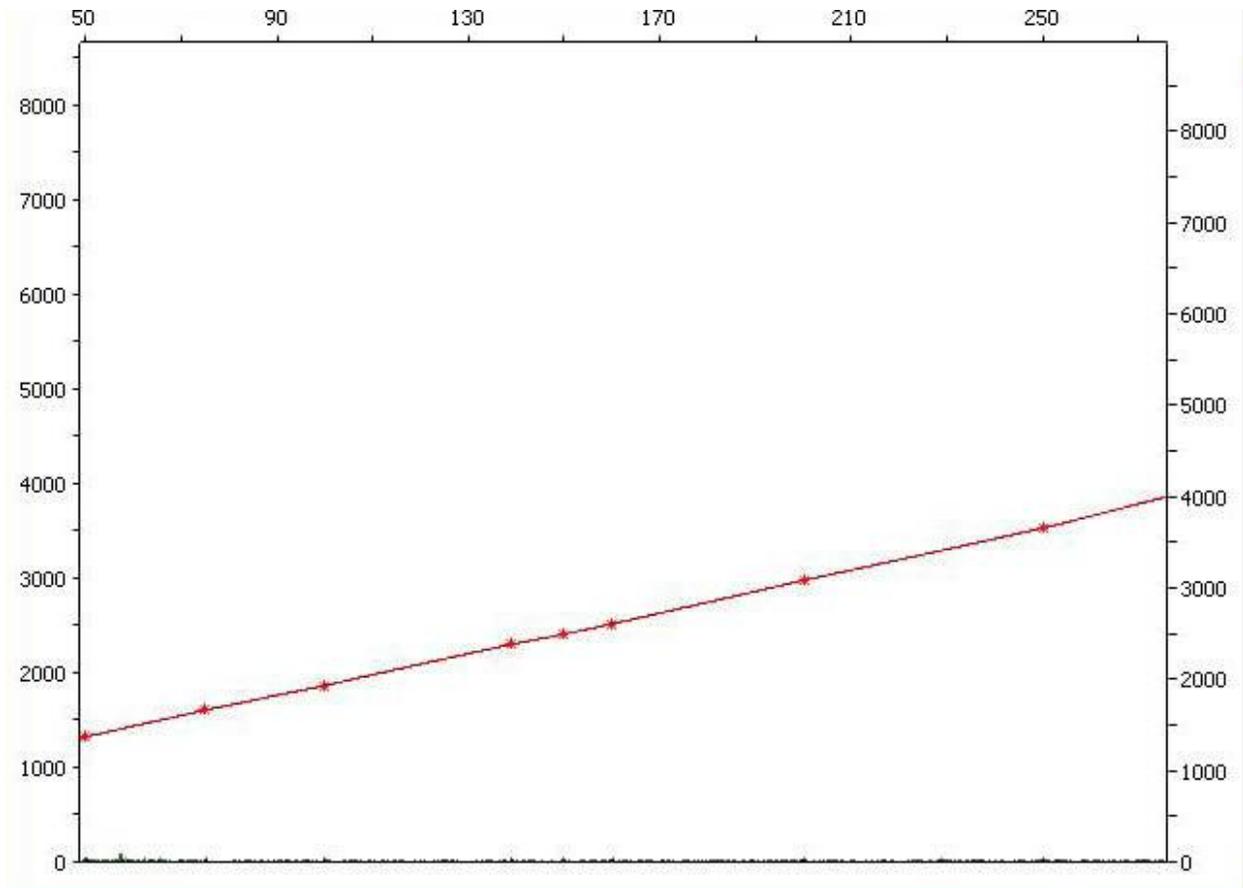
Figure 2: Graph representative of a sample that failed to produce any peaks at the PcLX16 and

PcI16 loci. Graph created using Peak Scanner (Applied Biosystems Inc.).

Figure 3: Graph representative of a successful analysis at the PcLX16 (with VIC florescent tag)

loci.   A)   represents allele at PcLX16 loci.   B) represents background noise caused by non-

specific product. Graph created using Peak Scanner (Applied Biosystems Inc.).