

A POPULATION DIVIDED: RAILROAD TRACKS AS BARRIERS TO GENE FLOW IN AN ISOLATED POPULATION OF MARBLED SALAMANDERS (*AMBYSTOMA OPACUM*)

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Abstract.—Habitat fragmentation reduces gene flow between isolated populations, thus increasing the risk of extinction through reduced genetic diversity due to the possibility of inbreeding and genetic drift. Ambystomatid salamanders are known to have limited vagility and high breeding site fidelity, potentially making them especially prone to negative effects of fragmentation. We compared gene flow between two populations of Marbled Salamanders (*Ambystoma opacum*) that are separated by railroad tracks. We additionally compared the genetic diversity of one population across two consecutive years. Observed heterozygosities within sites (0.60–0.65) were significantly lower than expected from the Hardy Weinberg Equilibrium, indicating that the populations may be inbred. F_{st} values and assignment test results corroborated the interpretation of semi-isolated populations. There was a greater difference in pairwise F_{st} between the populations on either side of the railroad tracks than between years on one side of the tracks. Assignment tests showed that > 60% of individuals were assigned as residents with high likelihood; whereas, only two individuals at each site were identified as immigrants. Our data suggest that the railroad track may act as a barrier to gene flow in these two populations.

Key Words.—*Ambystoma opacum*; amphibian; barrier; genetic diversity; Marbled Salamander.

INTRODUCTION

There is overwhelming evidence that habitat loss, fragmentation and degradation are primary factors in amphibian declines (Linder et al. 2003; Stuart et al. 2004; Cushman 2006). Fragmentation of habitat (change from continuous habitat to discreet habitat units separated by unsuitable habitat) can interrupt historic patterns of connectivity between subpopulations. Different species may be susceptible to differing levels of disruption based on dispersal ability (e.g., Hoehn et al. 2007). Although it has been clearly demonstrated that habitat loss and fragmentation are a serious threat to amphibian populations, there is little species-specific information currently available (Cushman 2006). The Ambystomatidae are of particular conservation interest, as this is the only salamander family with more rapidly declining species than the average for all amphibians (Stuart et al. 2004).

Ambystomatid salamanders are vernal pool breeders and are usually found in forested areas where multiple breeding pools exist. Marbled Salamanders (*Ambystoma opacum*) are known to return to their natal pools, with only 3.5–9% seeking new breeding areas (Scott 1994; Gamble et al. 2007). Their site fidelity is an additional impetus to identify and maintain successful breeding habitat, as Ambystomatids are unlikely to change breeding areas, even in the event of habitat degradation (e.g., Petranka et al. 2004). Additionally, understanding connectivity between populations is important in

developing conservation and monitoring strategies, especially in fragmented regions where several breeding pools may be found clustered within a woodlot.

Although Ambystomatid salamanders are known to return to their natal pool to breed, this generally does not appear to be sufficient to cause population differentiation in breeding populations less than 1 km apart. Zamudio and Wicczorek (2007) found that populations of Spotted Salamanders (*A. maculatum*) in a forested area within 4.8 km of each other showed evidence of genetic spatial autocorrelation. Purrenhage et al. (2009) found no evidence of isolation by distance in their study of this species. Likewise, Storfer (1999) found gene flow to occur in populations of Streamside Salamanders (*A. barbouri*) less than 5 km apart. Spear et al. (2005) found that gene flow did not drop off until distances greater than 1 km in Tiger Salamanders (*A. tigrinum melanostictum*) in Yellowstone National Park, Wyoming, USA.

Our goal was to examine the effect of an anthropogenic barrier on connectivity between geographically close populations of Marbled Salamanders (*A. opacum*) in southwestern Ohio. We compared genetic diversity and a genetic metric of isolation in populations of Marbled Salamanders on either side of a railroad track in an isolated woodlot. We compared these data to genetic diversity of a population on one side of the railroad track across two years. Due to their close proximity, we expected the populations of

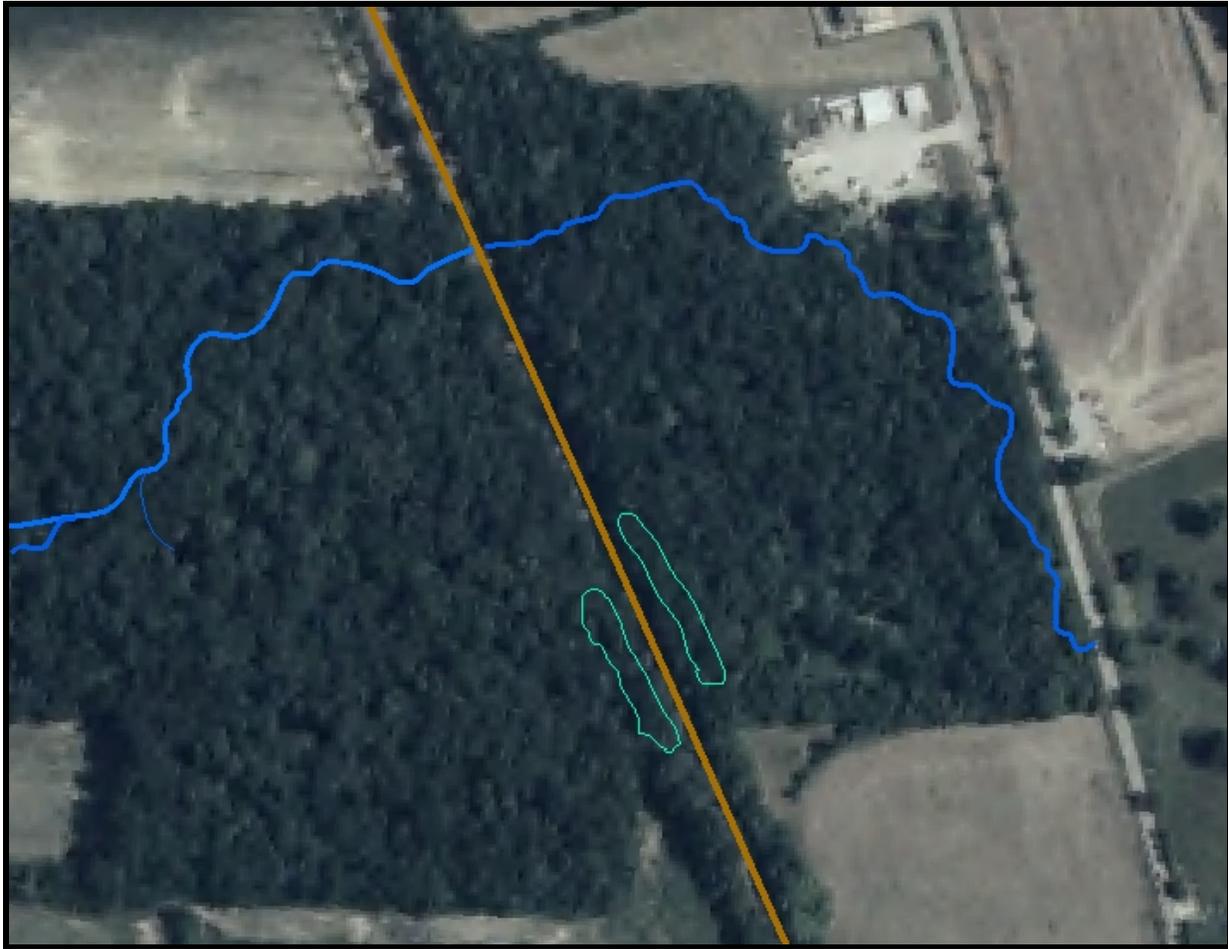


FIGURE 1. The east and west breeding pools of Marbled Salamanders (*Ambystoma opacum*) at a site in southwestern Ohio are outlined in light blue. A small stream (in dark blue), initiated by agricultural drainage tiles, flows from the west, under the railroad tracks (indicated by the brown line), to the north and east of the breeding ponds. The 17 ha woodlot is surrounded by agricultural fields, residential areas, and light industry.

Marbled Salamanders to be genetically similar despite the anthropogenic barrier.

MATERIALS AND METHODS

Study site.—The 17 ha woodlot is located in northern Hamilton County, Ohio, approximately 29 km northwest of Cincinnati, Ohio, USA. The woodlot is surrounded by agricultural, residential, and industrial property. A railroad bisects the woodlot into a 5.5 ha East and 11.5 ha West woodlot. Grading for the railroad track was completed in 1902 and the track was laid in 1903 (Taylor, R. (Ed.). 2003. *A Crosby Township History*. Crosby Township Historical Society, Crosby Township, Ohio.). There is a small stream that passes through the forested area to the north of the breeding areas and passes under the railroad tracks through a culvert (Fig. 1). Other Ambystomatid salamanders use this breeding area, including Spotted, Jefferson, and Smallmouth

Salamanders (*A. maculatum*, *A. jeffersonianum*, and *A. texanum* respectively).

No known Marbled Salamander breeding areas lie closer than Fort Ancient State Park, in Warren County, Ohio, approximately 54 km to the east, and East Fork Lake State Park, approximately 57 km to the southeast in Clermont County, Ohio. A population (35 km to the west) known historically from the southwest corner of Warren County, Ohio was apparently destroyed by commercial development; Kings Auto Mall (Jeff Davis, pers. comm.; King 1935). The surrounding landscape is not suitable habitat for Marbled Salamanders, being primarily agricultural fields, mixed with some rural residential and light industrial areas.

We placed funnel traps during the spring following the methods and protocol of Micacchion (Micacchion, M. 2002. Amphibian Index of Biotic Integrity (AmphIBI) for Wetlands. Final Report to USEPA, Testing biological metrics and development of wetland assessment

TABLE 1. Number and frequency of alleles across all populations of Marbled Salamanders (*Ambystoma opacum*) per locus. The lowest number of alleles at a locus was 6 (AmaD328) and the frequency of the most frequent allele was 42% (Aop36).

Locus	# of alleles	% most frequent
Aop31	11	27
AmaD328	6	38
AjeD162	11	28
Aop36	8	42
AmaD95	14	22
AjeD23	8	35

techniques using reference sites: Volume 3. Ohio Environmental Protection Agency, Division of Surface Water, Columbus, OH USA.), and captured larval Marbled Salamanders at each site. We collected 20 larvae from the east side of the railroad tracks in 2006. From the west side of the tracks, we collected seven individuals in 2005 and 10 individuals in 2006.

We used Qiagen DNeasy© kits (Qiagen, Valencia CA) to extract DNA from tissue. Individuals were genotyped at six microsatellite loci using locus-specific primers and the PCR protocol described by Croshaw et al. (2005). Two of the primers were developed for *A. opacum* (Aop31, Aop36), with an additional two each developed for *A. maculatum* and *A. jeffersonianum* (AmaD95, AmaD328, AjeD23, AjeD162). All microsatellites (non-coding tandem repeat units of DNA) were tetranucleotide repeats and the alleles were designated by four base pair differences in size. We used fluorescent-tagged forward primers to visualize alleles. We scored alleles using an Applied Biosystems 3100 automated sequencer and GeneMapper 3.2 software (Applied Biosystems, Foster City, California, USA).

We checked for deviations from Hardy-Weinberg Equilibrium (HWE) by performing exact tests in both HW-QuickCheck (Kalinowski 2006) and GENEPOP 3.4

(Raymond and Rousset 1995). We ran Micro-Checker 2.2.3 (van Oosterhout et al. 2004) to assure there were no null allele issues. We tested for genetic differences using two analyses available in GENEPOP 3.4, allelic distribution and genotypic distribution. Allelic distribution tests the null hypothesis that “the allelic distribution is identical across all populations.” The software constructs a contingency table for the alleles at each locus, each row representing a population, each column an allele. An unbiased estimate of the *P*-value for rejecting the null hypothesis is computed using a Markov chain method. Likewise, genotypic distribution tests the null hypothesis that “the genotypic distribution is identical across all populations.” Inbreeding coefficients (*F*-statistics) were calculated using the methods of Weir and Cockerham (1984) in GENEPOP 3.4 and Hedrick (2000) in GenAlEx 6 (Peakall and Smouse 2006).

We performed assignment tests in GeneClass2 (Piry et al. 2004) to determine the number of individuals of recent immigrant ancestry at each site. We used the “detection of first generation migrants” function with a Bayesian computation method (Rannala and Mountain 1997) and Monte-Carlo resampling (Paetkau et al. 2004) assuming a conservative alpha value of 0.01. High numbers of correct assignments (i.e., individuals sampled in population A and genetically assigned to that population with high likelihood) indicate that populations are differentiated (migration is rare), and provide an index of population isolation (Greenwald et al. in press).

RESULTS

Microsatellite loci were highly polymorphic. Across both populations the number of alleles varied from six to fourteen per locus with allele frequency of the most common allele being 22–42% (Table 1). Deviations from Hardy Weinberg Equilibrium (HWE) were tested

TABLE 2. Inbreeding coefficients (pairwise *F*-statistics) comparing both the West/East populations and the West population of Marbled Salamanders (*Ambystoma opacum*) between 2005/2006. Data are shown for each locus and for all loci combined (GENEPOP 3.4).

Locus	West/East		
	Fwc(is)	Fwc(st)	Fwc(it)
Aop31	0.1823	0.0316	0.2082
AmaD328	0.2452	0.0561	0.2875
AjeD162	-0.0315	0.0102	-0.021
Aop36	0.6826	-0.0156	0.6776
AmaD95	0.3482	-0.003	0.3463
AjeD23	0.0259	0.0432	0.068
All:	0.2357	0.0203	0.2512
Locus	West 2005/2006		
	Fwc(is)	Fwc(st)	Fwc(it)
Aop31	0.3132	-0.0524	0.2772
AmaD328	0.2805	-0.0378	0.2534
AjeD162	0.0602	-0.0453	0.0176
Aop36	0.6437	-0.0257	0.6345
AmaD95	0.401	-0.0603	0.3649
AjeD23	0.039	-0.0213	0.0185
All:	0.281	-0.0404	0.2519

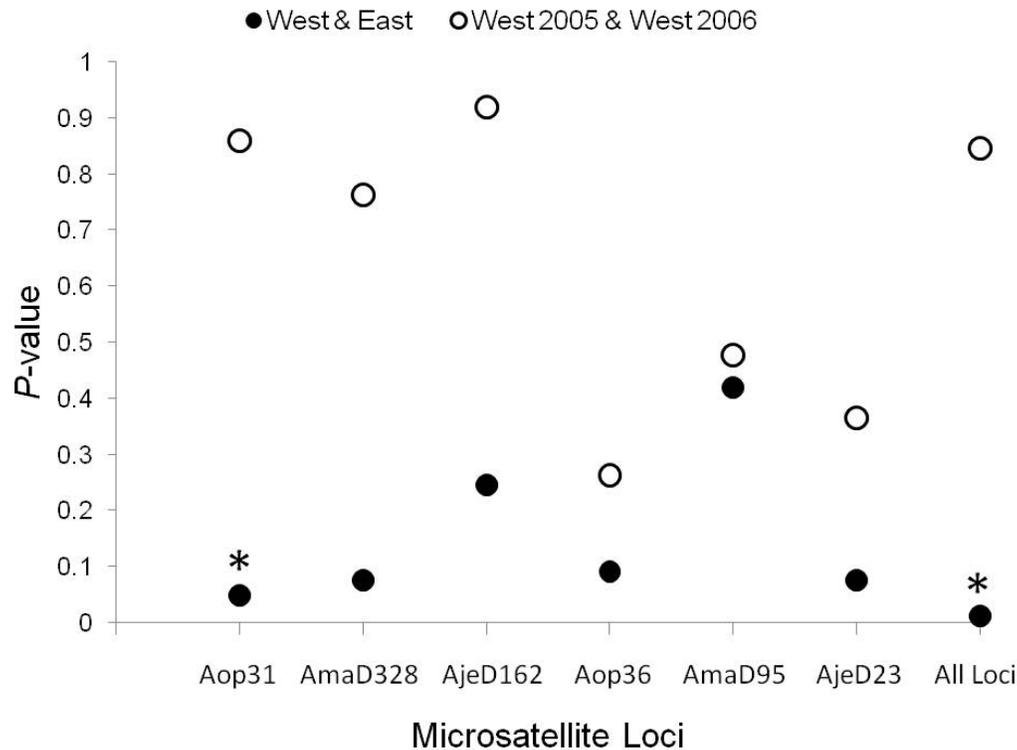


FIGURE 2. Genic (allelic) differentiation for population pairs of Marbled Salamanders (*Ambystoma opacum*). West and East comparison (filled circles), and West 2005 and West 2006 comparison (hollow circles), showing the P -value for each locus and for all loci using GENEPOP 3.4. The null hypothesis tested is H_0 : the allelic distribution is identical across populations. The difference between the West and East populations is significant ($P < 0.05$) but not between the two years on the West side ($P = 0.85$). Standard error bars are not shown as they were extremely small (mean SE = 0.005). Asterisk (*) indicates significance at $P < 0.05$.

and both populations deviate from HWE and have an excess of homozygotes. When we checked for null alleles, we found that no locus had evidence of scoring errors due to stuttering and no evidence for large allele dropout. In a few cases, null alleles were suggested by the general excess of homozygotes at a locus. In cases where adjusted allele frequencies were calculated, we ran all analyses with adjusted allele frequencies with no appreciable change in results.

Testing allelic distribution, the combined P value scores for West and East populations were significant ($P < 0.05$); however, the combined P value scores for the West 2005 and West 2006 populations were not significant ($P = 0.85$). This result indicates that the allelic distribution is different between the West and East populations, but not between the two years in the West population (Fig. 2). Although neither result was significant for the genotypic distribution, the results suggest there was more genotypic variation between the East and West populations than between the West 2005 and West 2006 populations ($P = 0.21$ vs $P = 0.97$, respectively).

Inbreeding coefficients (pairwise F -statistics) were compared both between the West and East populations

and between years (2005 vs 2006) in the West population (Table 2). Comparison of F_{st} values between years in the West and between the West and East populations demonstrate a greater difference between the populations on either side of the RR tracks than found between years on one side of the tracks (West vs. East: $F_{st} = 0.02$; West 2005 vs. West 2006: $F_{st} = -0.04$, equivalent to zero). Similarly, there is no molecular variance between the West 2005/2006 populations but a 3% molecular variance between the West and East populations.

Assignment tests revealed that a high proportion of individuals were assigned as residents of the pond at which they were caught. For the West population, 11 out of 17 individuals (65%) were assigned as residents with $> 95\%$ likelihood, while two individuals were assigned as immigrants (with 81.5 and 85.6% likelihood). For the East population, 12 out of 20 individuals (60%) were assigned as residents with $> 95\%$ likelihood, while two individuals were assigned as immigrants (with 78.1 and 83.6% likelihood). The remaining four (West) and six (East) individuals were assigned as residents with $< 95\%$ likelihood.

DISCUSSION

The major result of our work is that Marbled Salamander larvae in the West and East ponds exhibited significant genetic differentiation; whereas, those collected between years on the West side did not. Assignment tests revealed a relatively large proportion of individuals (> 60%) assigned to their population of origin with high certainty, also supporting the conclusion that these populations have been genetically subdivided by the railroad track (were there no subdivision, individuals would be equally assigned to both "populations" with low likelihood). These results are surprising, as review of an early land survey (Anon. 1819. Description and Notes of the Miami Survey. Hamilton County Parks, Hamilton County, OH USA.) suggests a single large wetland area was at this location until the grading for the railroad took place in 1902 (Innis 2003). Until construction of the railroad, it is likely that there was only one population in a single pond. The East and West populations are in close proximity and are potentially connected via movement both over the railroad tracks and along the stream through a culvert under the tracks. This configuration would appear to allow dispersal of individuals from one side of the tracks to the other relatively easily.

The moderate F_{st} value between East and West ponds indicates some gene flow across the tracks or may be a result of the recent division of a single population into two. Assignment tests support that some gene flow still occurs, as two individuals from each population were genetically assigned to the opposite side of the railroad track. The F_{st} value and assignment test results are comparable to those seen in other studies of Ambystomatids living in moderately fragmented habitats. In another study of Marbled Salamanders in southeast Ohio, pairwise F_{st} values ranged from 0.002 (where there was contiguous forest between sites that were 3 km apart), to as high as 0.217 (where there were significant barriers to movement between sites that were 13 km apart; Greenwald et al. in press). Resident assignment in the same study ranged from 16-100% of individuals. In Yellowstone National Park in Wyoming, Spear et al. (2005) found F_{st} values were 0.026 and 0.010 in populations of Tiger Salamanders <1 km apart, indicating there was no significant differentiation between the populations at this distance. Across his study area, the salamanders had an F_{st} of 0.24. Amphibians, generally, have higher F_{st} values than other taxa ($F_{st} = 0.315$ for 33 species of amphibians, versus $F_{st} = 0.076$ for 16 species of birds; Ward et al. 1992). This is likely due in large part to the generally low dispersal ability and high philopatry of amphibians. The F_{st} between the two sides of the railroad track, while low in absolute terms, is higher than expected for ponds in such close proximity. This result is striking when compared

with values between years on one side of the railroad tracks, and with values seen in Marbled Salamander populations in other parts of Ohio.

Observed heterozygosities (approximately 0.60 to 0.65) in our populations were consistently far below expected heterozygosities (0.96), a deviation from Hardy Weinberg Equilibrium (HWE). A population should attain and remain in HWE after a single generation if certain assumptions (a large, sexually reproducing population with random mating, and no selection, mutation, or migration) are not violated. Deviations from HWE are probably due to the small size and genetic isolation of this population. Small populations also tend to have higher F_{is} values (inbreeding coefficient for subpopulations). These Marbled Salamander populations have high (> 0.2) inbreeding coefficients as compared with other studies (e.g., -0.089–0.218, Spear et al, 2006); the high F_{is} values and deviation from HWE may indicate that this isolated population of Marbled Salamanders is under genetic stress.

The populations have been separated by the railroad for a little over 100 years or about 25 overlapping generations, given that the average age to sexual maturity in this species is 3–4 years (Pechmann 1995). However, our results indicate that the same animals (or their offspring) breed on the west side of the railroad tracks in subsequent years, and that animals breeding on the east side of the railroad tracks have different alleles than those that breed on the west side. This site fidelity has led to genetic subdivision despite the relatively short time since the ponds were isolated, and also despite mechanisms that might facilitate gene flow, such as female sperm storage (Krenz and Scott 1994).

Numerous studies have shown that anthropogenic habitat modification can lead to changes in occurrence, behavior, and population genetic structure. Marbled and Spotted Salamanders are known to enter and exit breeding areas in a non-random manner (Shoop and Doty 1972) preferring forested to field conditions and showing increased rates of desiccation in field over forest conditions (Rothermel and Semlitsch 2002; Rothermel et al. 2008). Roads have been shown to reduce anuran species density (Eigenbrod et al. 2008) as well as Tiger Salamander pond occupancy (Porej et al. 2004). Gene flow is restricted by roads in ground beetles (Keller and Largiader 2003), and gecko species sensitive to inhospitable habitat have lower genetic diversity than those that are less sensitive (Hoehn et al. 2007). Our study adds to the body of literature supporting the conclusion that even relatively narrow anthropogenic barriers (i.e., the railroad track) can lead to isolation of affected populations.

The forested area around the breeding pools appears to be large enough to sustain a population of salamanders. The West woodlot is 11.5 ha (~ 400 m from the pond to

the furthest forest edge) and the East woodlot is 5.5 ha (~ 200 m from the pond to the furthest forest edge). Semlitsch and Bodie (2003) suggest that a buffer of 218 m from the edge of the breeding pool would protect sufficient upland habitat to support a breeding population of salamanders, and that 142–289 m around the pond should be considered core habitat. Adult Marbled Salamanders migrate an average of 194 m (range: 0–450 m) in Indiana (Williams 1973); however, they have been found to migrate over 1 km in other systems (Gamble et al. 2006). Our site is likely a self-sustaining population because the closest known breeding population is more than 50 km away, much farther than these animals are known to migrate. Additionally, the land area between this population and the other known populations would be hostile for any migration attempt.

Genetic analyses (allelic distribution, genotypic distribution, F_{st} values, and assignment tests) suggest that gene flow between the breeding ponds on either side of the railroad tracks is low. Comparing F_{st} and assignment tests of Marbled Salamanders in southeast Ohio with measures for these populations suggest the railroad tracks act as a moderate barrier to gene flow. In this case the construction of the railroad appears to have further fragmented the habitat within an isolated woodlot. High F_{is} and deviation from HWE suggest these populations may already be experiencing reduced genetic diversity. Barriers to gene flow like the railroad track can amplify genetic stress. Conservation action should focus on maintaining and enhancing connectivity between populations of Marbled Salamanders in order to maintain genetic diversity in these populations.

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