Effect of fragmentation on isolation of salamanders in Michigan's Lower Peninsula

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Abstract

The growing human population is impacting the natural landscape in many ways, including increased destruction of, and fragmentation of, habitat. The building of roads has cut up continuous habitat into smaller patches, out of which animal movement is unlikely. For wildlife, and specifically amphibians who are already in global decline (Alford and Richards 1999; Blaustein et al 1994), increasing habitat fragmentation will further damage populations. Salamanders rarely cross roads and many species require ponds for breeding, making movement between populations particularly difficult in a fragmented landscape. Movement of individuals between neighboring populations is important because it allows populations to retain genetic diversity and avoid inbreeding. In order to measure the genetic effects of fragmentation in Michigan, DNA was collected from salamanders at the Pierce Cedar Creek Institute (Hastings), Vestaburg Bog (Vestaburg), and Neithercut Woodlot (Farwell). Genetic analyses were used to determine population structure at three levels: Southern Michigan (Pierce Cedar Creek Institute), Mid-Michigan (Vestaburg and Neithercut), and southern half of the Lower Peninsula (all 3 sites combined). The relatedness of salamanders from each site was compared, as the differences in distance between them will provide 3 scales for analysis; ~50 km (Vestaburg-Neithercut), ~100 km (Vestaburg- Pierce Cedar Creek Institute) and ~150 km (Farwell- Pierce Cedar Creek Institute). There are two genetically distinct populations of redback salamanders: mid-Michigan containing both Vestaburg and Neithercut redbacks

and southern Michigan containing the Pierce Cedar Creek Institute salamanders. These populations are likely isolated by a geographic barrier such as road densities, I-96, or the Grand River rather than by distance.

Introduction

Habitat fragmentation and destruction are increasing as the human population grows and spreads across the landscape. Urban sprawl is a growing problem in the U.S., especially in mid and southern Michigan where undeveloped gaps between Chicago and Detroit are quickly filling (Miller 2004). Knutsen et al. (1999) found an inverse relationship between population size and abundance of urban land, and a positive relationship between population size and abundance of forest and wetland habitats. Fragmentation negatively affects population densities of species by reducing available habitat and isolating species unlikely to cross unsuitable habitat matrix (Tischendorf et al. 2005). Habitat fragmentation contributes to the global decline of amphibians (Alford and Richards 1999; Blaustein et al 1994).

One major force fragmenting forest habitat and isolating species is the building of roads (Marsh et al. 2005). For every linear kilometer of road approximately 4 hectares of forest is lost (deMaynadier and Hunter 2000). In addition to actual forest lost, edge effects prevent some species, such as *Ambystoma* salamanders, from using a large buffer region adjacent to roads (deMaynadier and Hunter 1998). The reduced patch size associated with fragmentation also reduces the population size found within the fragments (Marsh and Pearman 1997). Roads have cut up once continuous habitat into smaller patches of habitat, out of which dispersal is unlikely.

Genetic exchange between neighboring populations maintains allelic diversity in those populations. Isolated populations become inbred which elevates extinction risk (Frankham 1995; Frankham 1998, Newmark 1996) and exacerbates the negative effects of bottlenecks (Andersen et al. 2004). Bottleneck events reduce the number of breeders in a population and remove rare alleles (Luikart et al. 1998; Bellinger et al. 2003; Charlesworth et al. 2003). These factors ultimately reduce individual fitness (Bellinger et al. 2003) which further reduces population size, and exacerbates the inbreeding and bottleneck (Lacy 1997). However, even rare immigration can increase genetic variation and ameliorate the effects of inbreeding (Vilà et al. 2002).

Salamanders have limited dispersal distances increasing their likelihood of becoming isolated; *Plethodon cinereus* 90m , *A. laterale* 405m, *A. maculatum* 756m, and *A. tigrinum* 600m (Smith and Green 2005). *Ambystoma* have greater maximum dispersal distances than *Plethodon* but are more limited by their habitat requirements; bottomland deciduous and coniferous forests, woodlands, and vernal ponds for reproduction at snowmelt (Petranka 1998). These salamanders are more susceptible to isolation because of their dependence and fidelity to ponds (Marsh and Trenham 2001; Smith and Green 2005) and their reluctance to disperse across unsuitable habitat (Rothemel and Semlitsch 2002). Woodland salamanders (genus: *Plethodon*) are smaller than the *Ambystoma,* terrestrial in all life stages, and hatch fully developed. One plethodontid and one species of *Ambystoma* will be sampled for genetic analyses: *Plethodon cinereus* and *A. laterale*. We expected to find 3 populations of both species with gene flow between the populations being related to distance between them.

Methods

Redback salamanders were found by flipping logs at Pierce Cedar Creek Institute. Vestaburg Bog and Neithercut Woodland redbacks were collected and genotyped by Veverica and Swanson (2006). Blue spotted salamanders were collected using ~75-90 m of drift fencing and pitfall traps placed every ~9-15 m along the drift fence after snowmelt at Pierce Cedar Creek Institute and Neithercut Woodland. The distal 2-5mid-Michigan of the tail was clipped with surgical scissors. Collected tissue was stored on ice in the field and moved to -80˚C freezer in the lab.

DNA was extracted using QAIGEN DNEasy Tissue Kits and following the published protocol (Qaigen 2001; Valencia, CA). We quantified and determined the purity of the extracted DNA using an Eppendorf Biophotometer (Brinkman Instruments Inc.; Westbury, NY). Half of each sample was diluted to a working stock of 15-ng/ µl and stored at 4°C, and the remainder was stored at -80°C.

All redbacks were genotyped at 7 microsatellite loci (PcLX23, PcJX24, PclI14, PcLX16, PclI16, PcXF08, PcJX05; Connors and Cabe 2003). PcLX16 was removed from analysis for comparison with Veverica and Swanson (2006) redbacks from Neithercut Woodland and Vestaburg Bog. All blue spotted salamanders will be genotyped at 8 microsatellite loci (AjeD23, AjeD75, AjeD94, AjeD108, AjeD283, AjeD346, AjeD422; Julian et al. 2003a; AmaD367; Julian et al. 2003b). All reverse primers were labeled fluorescently with FAM, TET, or HEX. PCR was conducted in a 20µl mixture of 75ng of template DNA, 2µl 10x HotMaster Taq Buffer, 1µl Bovine Serum Albumin (10mg/mL), 250 µM dNTPs, 0.16 µM of each primer, 0.20µl HotMaster Taq Polymerase (Brinkman Instruments Inc.; Westbury, NY), and ultrapure water to

complete the volume (Brinkman Instruments Inc. Westbury, New York). The PCR was run on an Eppendorf MasterGradient Thermocycler (Brinkman Instruments Inc.; Westbury, NY) for a 2 minute denaturation at 94°C followed by 30 cycles at 94°C for 30 seconds, 30 seconds at the appropriate annealing temperature (PcLX23 and PcJX24 at 60°C, PclI14 at 57°C, PcLX16 at 55°C, PclI16 at 58°C, PcXF08 at 56°C, PcJX05 at 51°C), and 30 seconds at 72°C. This finished with an elongation period at 72°C for 5 minutes (Veverica and Swanson 2006).

The PCR product was diluted 1:10 with ultrapure water. 1µl of the dilute PCR product was mixed with 12µl of formamide and 0.3µl of TAMRA 500 size standard and heat shocked at 95°C for 5 minutes and then placed immediately in to a freezer at -80°C. We then ran the heat-shocked products on an ABI 310 Automated DNA Sequencer (Applied Biosystems; Foster City, CA). Genotyper 2.0 was used to determine genotypes (Applied Biosystems).

The number of genetically distinct populations were estimated with Geneland (Guillot et al. 2005) a landscape genetics program which incorporates both spatial and genetic data. Geneland operates without *a priori* population assignment and utilizes Markov chain Monte Carlo to infer likelihood of assignment. The results are mapped and can be matched with physical features of the landscape. For the redbacks Geneland mcmcFmodel was run for 122 individuals at 6 microsatellite loci, minimum number of populations was 1, initial number of populations was 3, and the maximum number of populations was 5. The maximum number of nuclei was 366, and the program completed 50000 iterations with a thinning interval of 50. The freq.model was Dirichlet and varnpop equaled true. Geneland mcmcFmodel was run again with the npopmax and

npopinit set to 2 because the initial run indicated that 2 populations had the highest probability density. We ran the mcmcFmodel both with (delta.coord = 1000) and without spatial data (delta.coord=0).

The allele frequencies, F_{st}, and F_{is} were calculated with GenePop on the Web (http://wbiomed.curtin.edu.au/genepop/) using default parameters. The program Bottleneck (Corneut and Luikart 1996) was used to determine if a recent bottleneck occurred in the salamander populations. Bottleneck compares the heterozygosity of a population to the expected heterozygosity assuming mutation-drift equilibrium (Corneut and Luikart 1996). The presence of a genetic bottleneck within the last $0.5\ N_e - 5N_e$ generations is indicated if heterozygosity is significantly greater than that expected under drift-mutation equilibrium (Corneut and Luikart 1996). The Two-phase model of mutation was selected because it more closely models microsatellite mutation compared to the Infinite Allele Model and the Stepwise Mutation Model (Luikart et al. 1998). Bottleneck was used to perform the sign test, standard differences test, Wilcoxon sign rank test and the qualitative mode shift test all following 1000 iterations.

Doh (http://www2.biology.ualberta.ca/jbrzusto/Doh.php) was used to estimate movement between populations. Doh assigns individuals to populations based on the likelihood of their genotype occurring in the most likely population (Paetkau et al. 1995). Doh was run with 1000 randomizations and resampling without replacement at each locus within a sample site. Extremal statistics were used to determine significance levels for the likelihood of mis-assignment. If assigned to a population other than that of capture, then the individual is likely to be an immigrant.

Spatial data was collected from the Michigan Center for Geographic Information via the Geographic Data Library (http://www.mcgi.state.mi.us/mgdl). Road density in the study area was derived from Michigan Geographic Framework: State of Michigan (version 6b) of all roads in the Lower Peninsula. Study sites were identified using road data layers; a shapefile of these points was created. The road dataset was converted to raster data with 100m cells in ArcMap 9.0 for use in ArcView 3.2 (Figure 1; ESRI, Redland, CA).

Individual shapefiles were created connecting each pair of study sites (n=3) by a single straight line. Each of these straight line paths were buffered by 200 meters on both sides of the line (Figure 2; suggested seasonal movement of *A. maculatum*; Petranka 1998). Buffered paths were used to clip roads from the raster file in ArcView 3.2 (Figure 3). Areas of the buffered paths, length of path, and sum of road lengths within the clip were calculated using the Xtools extension in ArcView 3.3. Road density was calculated for each buffered path using the equation: $RoadDensity = \frac{\sum RoadLengths}{Area_{BufferedPath}}$.

Results

Sufficient numbers of redback salamanders were collected at all three sites for genetic analyses (Table 1). Due to the timing of blue spotted salamander migration to vernal pools a sufficient number of samples was able to be collected at Neithercut Woodland and Pierce Cedar Creek Institute, but not Vestaburg Bog (Table 1). Additional sampling efforts are needed to include Vestaburg in the analysis for blue spotted salamanders.

Geneland identified two populations of redbacks with 100% probability densities with spatial and genetic data together and genetic data alone (Figure 1). Neithercut Woodland and Vestaburg Bog compose the mid-Michigan population and Pierce Cedar Creek Institute belongs to a southern Michigan population (Figure 2).

There is no significant linkage disequilibrium between any of the 6 loci (all $p > 0.05$). The mid-Michigan population has one locus out of HWE, X05; while the southern Michigan population has 3 loci out of HWE, X23, I14, and X05. The allelic diversity (Figure 3) in mid-Michigan is significantly different from southern Michigan at three loci (X24, I16, X05; $p < 0.05$) following resampling statistics to correct for unequal sample sizes. Heterozygosity appears to be higher in the mid-Michigan population (Figure 4). Inbreeding coefficients for southern Michigan appear higher than mid-Michigan values (Figure 5).

The mid-Michigan population has a normal L-shaped distribution of allele frequencies (Figure 6) and fits the two-phased model of mutation ($p = 0.51$) and the Wilcoxon test ($p=0.69$). The expected number of loci with heterozygosity excess was 3.50 and the observed was 4 loci. The southern Michigan population also has a normal L-shaped distribution of allele frequencies (Figure 6) and also fits the TPM of mutation by the sign ($p= 0.48$) and Wilcoxon ($p = 1.00$) tests. The observed number of loci with heterozygosity excess was 3 and the expected number was 3.52. The assignment test identified only one significantly mis-assigned individual ($p = 0.012$). This redback was captured in the mid-Michigan population but assigned to southern Michigan.

Buffer area, total road lengths, and path length were greatest from Pierce Cedar Creek Institute -Neithercut (Table 2). The greatest road density was from Pierce Cedar

Creek Institute -Vestaburg followed by Pierce Cedar Creek Institute -Neithercut (Table 2). Vestaburg-Neithercut contains the lowest total road length and road density.

Discussion

There are two genetically distinct populations of redback salamanders. The allelic diversity within each population, mid-Michigan =6 southern Michigan =5.2, appears similar to an external study of 16 redbacks with a mean number of alleles equaling 6.1 (Connors and Cabe 2003). Average heterozygosity in both mid-Michigan (H=0.48) and southern Michigan (H=0.38) appears lower than what Connors and Cabe (2003) found (H=0.61). Both the mid and southern Michigan populations have lower allelic diversity and heterozygosity compared to other amphibian populations.

Andersen et al. (2004) found amphibian populations to be inbred at F_{is} values ranging from 0.198-2.65. By this metric the mid-Michigan population is not inbred (F_{is} =0.13), but the southern Michigan is inbred (F_{is} =0.37). However, neither population of redbacks showed evidence of a recent genetic bottleneck. A population reduction that decreased mating opportunities could result in an increase in inbreeding but not be strong enough to register as a bottleneck. This suggests that the high inbreeding coefficient in southern Michigan is likely due to isolation, limited immigration, and a possible population reduction.

Limited or no dispersal occurs between the mid-Michigan and southern Michigan populations. The single mis-assigned individual identified by Doh is likely a rare genotype in the mid-Michigan population and not an actual disperser or descendent of one. Even if this one individual were a disperser it still leaves southern Michigan isolated

from accepting dispersers because this individual was captured in mid-Michigan but genetically more similar to southern Michigan.

I-96 may be a barrier to dispersal between mid-Michigan and southern Michigan redbacks. When the contours of probability of assignment from Geneland are overlayed on a road map (Figure 7) I-96 bisects the space between the 0.5 and 0.6 contours. When the same contours are overlayed on a map of rivers the Grand River also bisects the space between the 0.5 and 0.6 contours running roughly parallel to the 0.5 contour (Figure 8) suggesting that this may be a natural barrier. Additionally, road densities are greatest from both Vestaburg and Neithercut south to Pierce Cedar Creek Institute indicating that the habitat may be too fragmented for salamander movement. Each of these hypothetical barriers requires further investigation before any management decisions are made.

Management Recommendations

To restore genetic diversity and reduce inbreeding in the southern Michigan population the barriers for dispersal must be positively identified. Knowing the barriers to movement, increased dispersal should be facilitated possibly by creating corridors or performing translocations. If the barrier is natural (ex: Grand River) then connecting the gene pools on both sides could result in outbreeding and a loss of local adaptations resulting in decreased fitness. If the barrier is anthropogenic (ex: I-96 or road density) then reconnecting those gene pools on either side of the barrier may result in reduced inbreeding and increased evolutionary potential. To maintain the stability of the mid-Michigan population the existing dispersal corridors should be identified and maintained.

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Table 1. Number of samples of redback and blue-spotted salamanders collected at PCCI, Neithercut, and Vestaburg.

	PCCI	Neithercut	Vestaburg
Redback	32	60	30
Blue-Spotted	32	34	8

Table 2. Road density metrics between the three sites including buffer area, total road length, road density and straight-line path length.

	PCCI- Neithercut	PCCI- Vestaburg	Vestaburg- Neithercut
Buffer Area (ha)	6083	2267	4071
Total Road Length (km)	458.5	283.2	148.2
Density (km/ha)	0.075	0.125	0.036
Path Length (km)	151	101	56

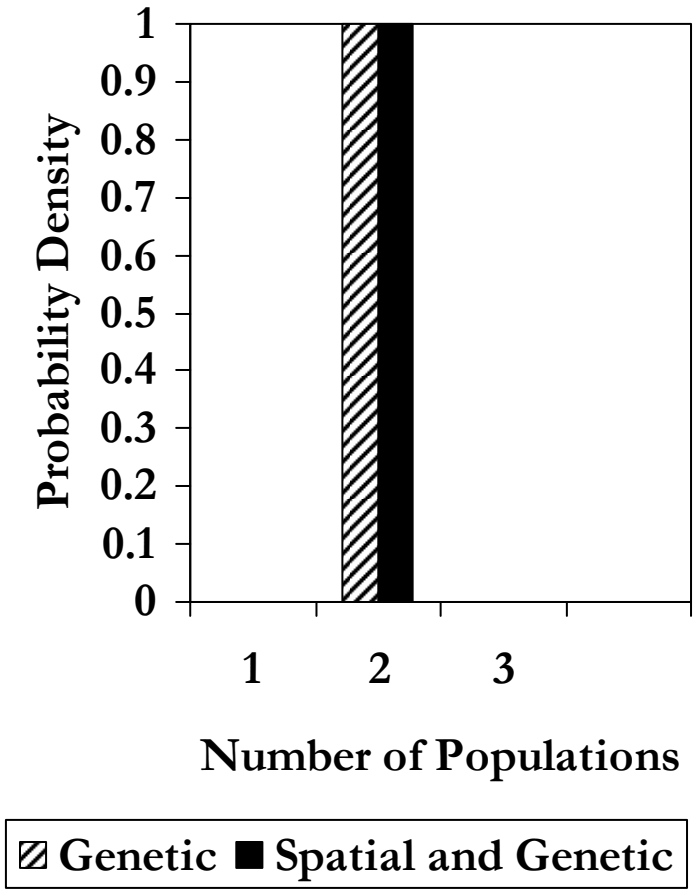

Figure 1. Number of populations of redback salamanders in Michigan's lower peninsula as calculated by Geneland (Guillot et al. 2005) with genetic data alone and spatial and genetic data together.

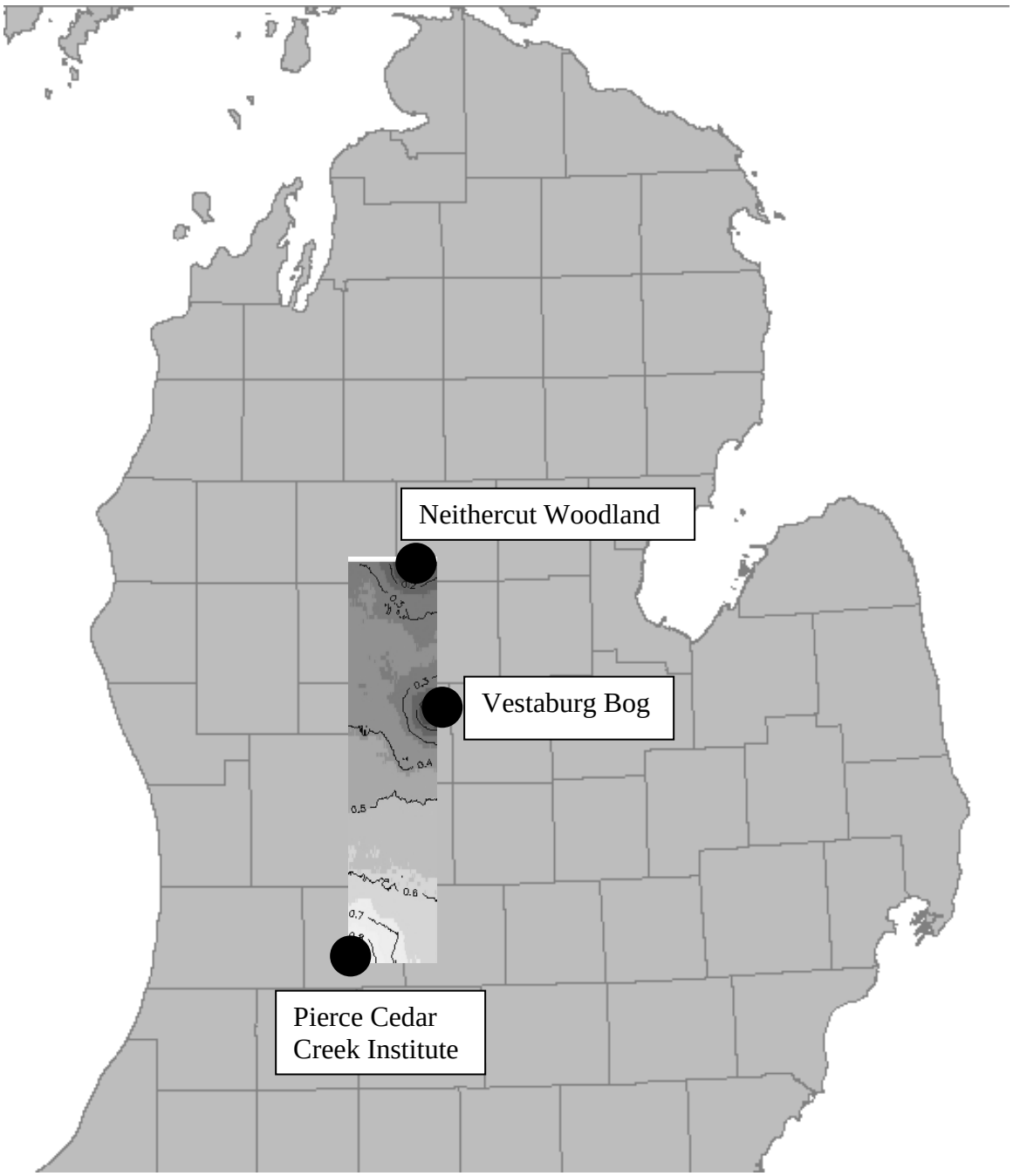

Figure 2. Geneland (Guillot et al. 2005) map of posterior probability of assignment to the southern Michigan population (Pierce Cedar Creek Institute) of redback salamanders.

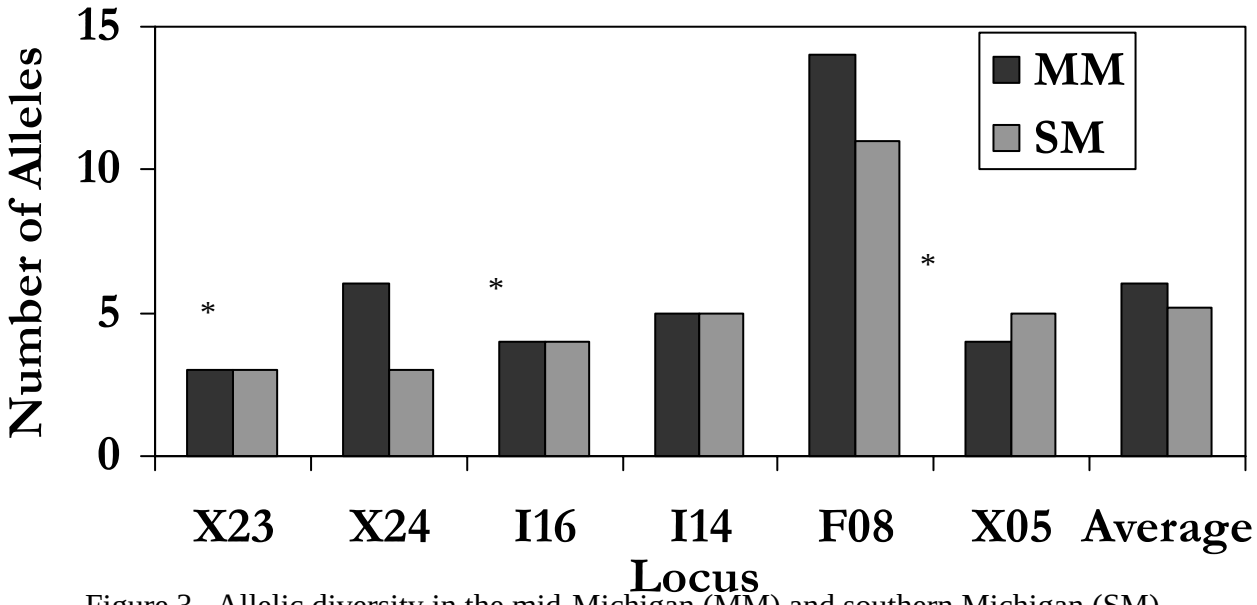

Figure 3. Allelic diversity in the mid-Michigan (MM) and southern Michigan (SM) populations of redback salamanders. *Indicates significant ($p < 0.05$) difference with resampling to correct for unequal sample sizes.

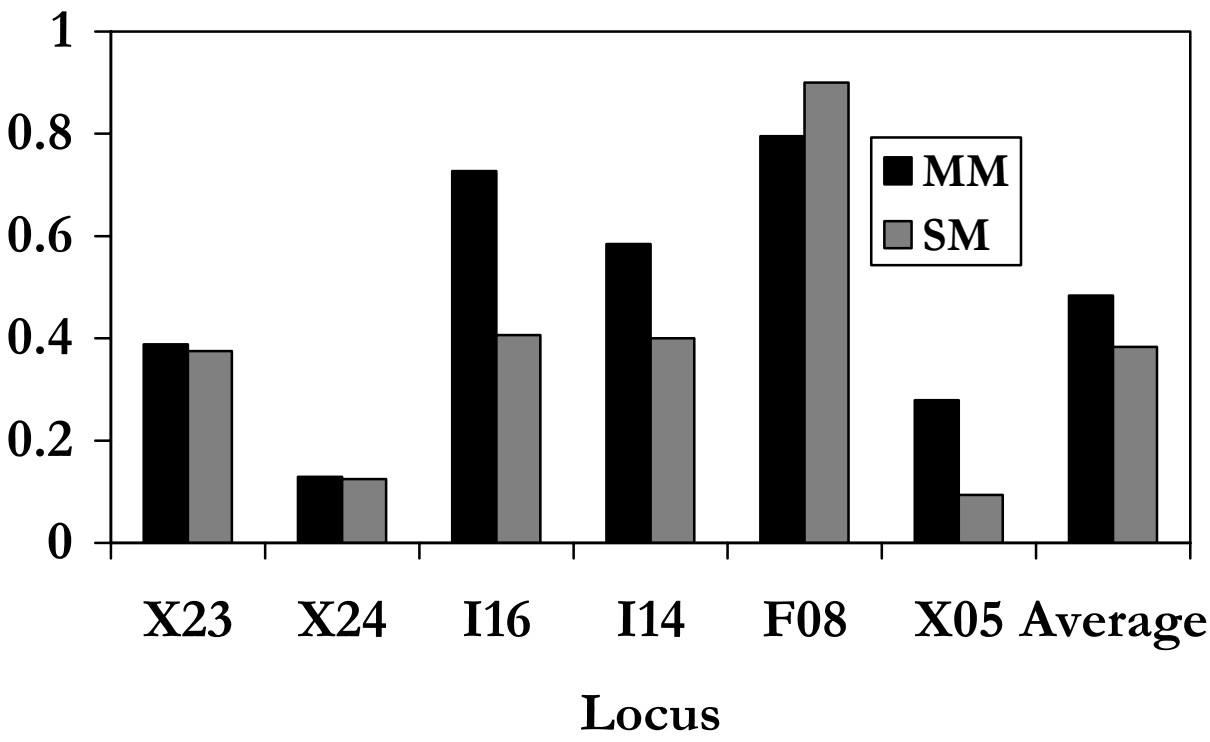

Figure 4. Heterozygosity in the mid-Michigan (MM) and southern Michigan (SM) populations of redback salamanders.

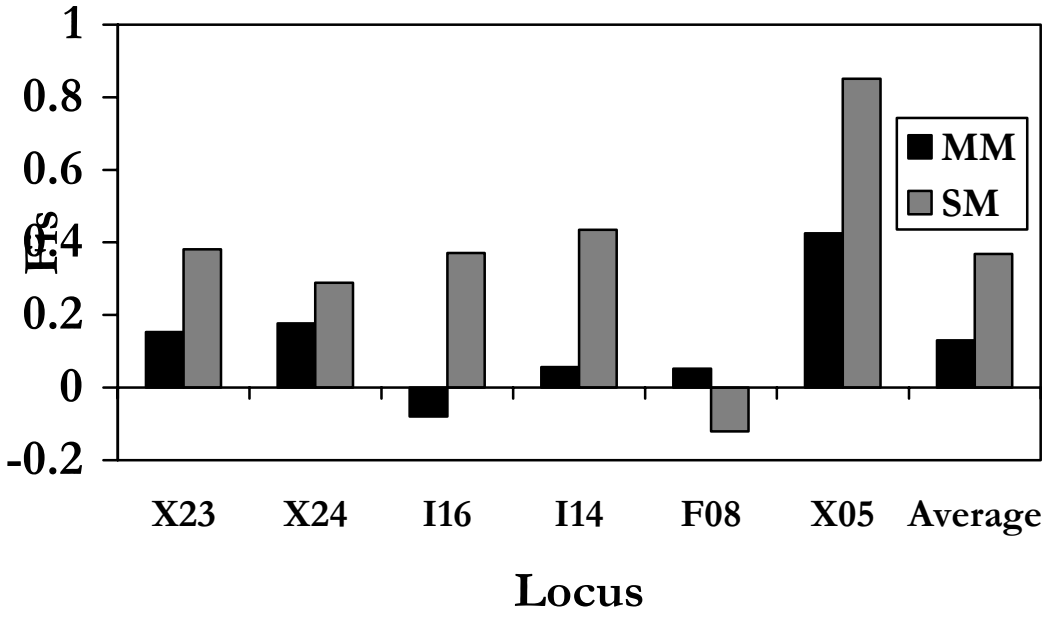

Figure 5. Inbreeding coefficients at each locus for the mid-Michigan (MM) and southern Michigan (SM) populations of redback salamanders.

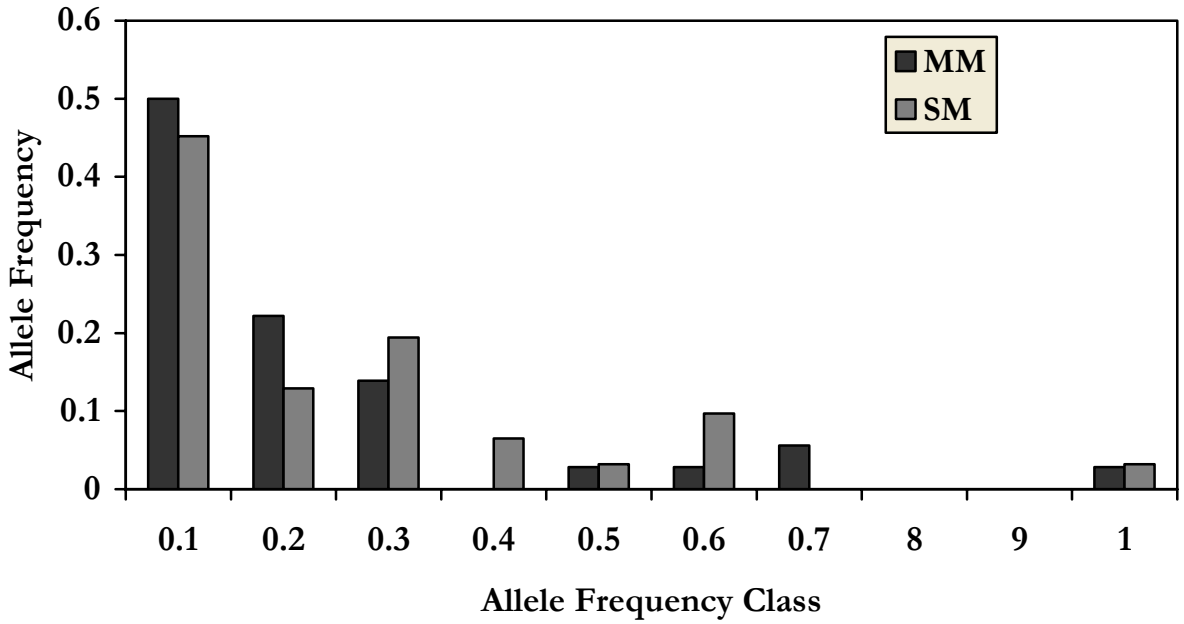

Figure 6. Allele frequency distribution of the mid-Michigan (MM) and southern Michigan (SM) population.

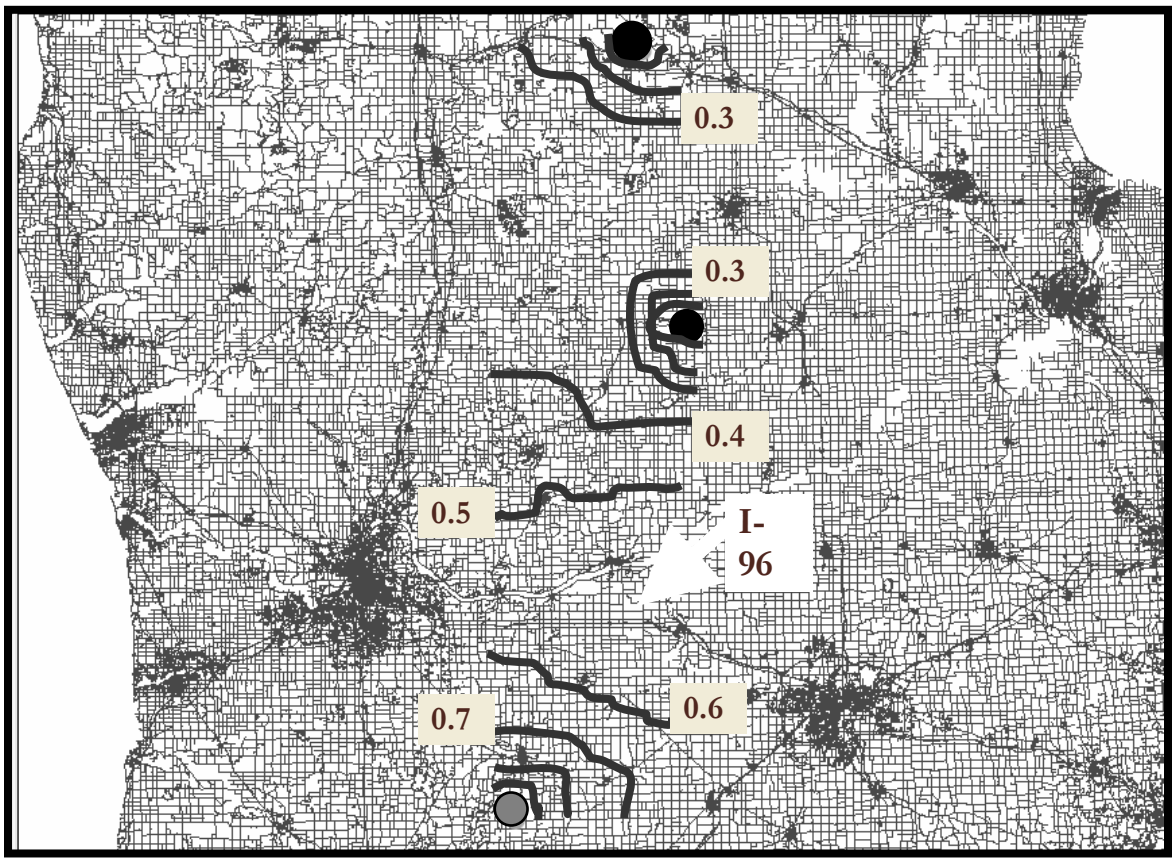

Figure 7. Probability of assignment contours from Geneland overlayed on road map. Mid-Michigan sample sites are represented as solid black circles, the southern Michigan site is a solid gray circle.

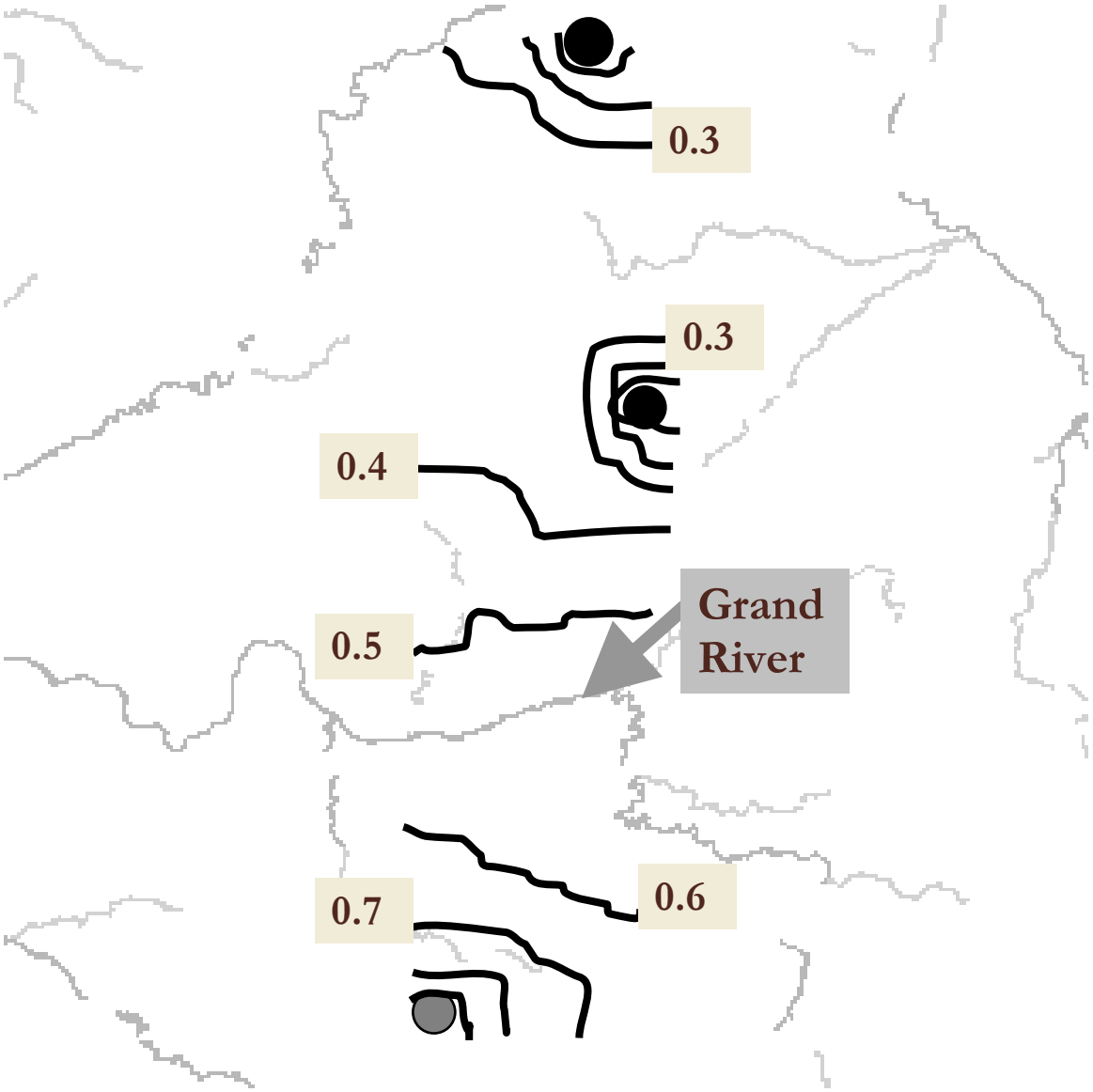

Figure 8. Probability of assignment contours from Geneland overlayed on river contours (light gray). Mid-Michigan sample sites are represented as solid black circles, the southern Michigan site is a solid gray circle