5-8-2017

Effects of Habitat Fragmentation on the Population Genetics of *Plethodon cinereus*

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Submitted in Partial Completion of the
Requirements for Departmental Honors in Biology

Bridgewater State University

May 8, 2017

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1. Abstract

Genetic diversity is important to the long-term survival of a species. Populations with low genetic diversity are more likely to go extinct due to an inability to adapt to environmental changes or accumulation of deleterious alleles. Habitat fragmentation may result in lower genetic diversity by disrupting gene flow between subpopulations caused by urbanization and development (e.g., roads, buildings, and agricultural fields). This study explored the effects of habitat fragmentation on the genetic diversity of the eastern red-back salamander (*Plethodon cinereus*). I collected tissue samples from 20-30 individuals from 1-3 different subpopulations within four larger forest sites that differed in their total area (i.e., >1000 hectares or 3-60 hectares). Two sites were small fragments that are completely surrounded by suburban or agricultural areas in eastern Massachusetts ("fragmented" forest; three subpopulations). The other two sites were large, continuous forests located in western Massachusetts ("continuous" forest; four subpopulations). DNA was extracted and amplified at five to seven microsatellite loci. I calculated heterozygosity, allelic richness, and overall population differentiation. Two out of three fragmented subpopulations had significantly lower than average allelic richness, while one out of four continuous subpopulations had significantly higher than average allelic richness. I also found stronger differentiation (higher F_{ST}) between fragmented subpopulations than expected from simply isolation-by-distance, and evidence of secondary structure between the fragmented sites. This suggests that while genetic diversity in this species may not yet be directly affected by habitat fragmentation, dispersal is limited between fragmented subpopulations (reflected in their higher F_{ST} compared to continuous subpopulations).

2. Introduction

2.1 Habitat Fragmentation: Causes and Genetic Consequences

Habitat degradation and fragmentation threaten species due to overall habitat loss and the creation of barriers to dispersal and migration (Cushman 2006, IUCN 2017). Habitat loss is thought to be the most important driver of extinction (IUCN, 2017), and has become increasingly common as humans use and develop greater amounts of landscape. Habitat degradation occurs directly through land development, when construction occurs on or in the habitat, or indirectly as a result of changed ecosystem functions like water flow and nutrient run-off (Hanski 2015.). Habitat fragmentation occurs when changes in land use fragment, or break apart previously continuous habitats with uninhabitable developed space. New anthropomorphic landscape features, such as settlements and roads, act as barriers between the previously connected natural habitats. The barriers separate populations in these habitats from each other and limit their access to resources across the landscape, leading to greater competition within the smaller habitat fragments, disruption of migration, and negative genetic effects (Hanski 2015). It is these genetic effects that this study is most concerned with.

There are several ways in which fragmentation can directly affect the genetics of populations living within previously continuous habitats. First, roads and other developments act as barriers for individuals, and limit the migration and gene flow between populations in different fragments (e.g., Marsh et al. 2008; Marsh et al. 2004b). Limited migration combined with a reduced habitat size may lead to decreased genetic diversity: migrants rarely or never introduce new alleles, and fewer individuals supported by the smaller habitat means fewer mutations occur each generation (Freeland et al. 2011). The loss of genetically diverse migrants and reduced habitat size may increase species extinction risk, as populations may lack the

diversity required for a future change in selection pressures (e.g., Saccheri et al. 1998; Puttker et al. 2015).

Second, smaller, less genetically diverse populations found in fragments also may have higher rates of inbreeding, which further increases homozygosity (Freeland et al. 2011). Inbreeding occurs in greater frequency when populations are small, as the cost of not finding a mate outweighs the cost of mating with a genetically similar individual (Lohr and Haag 2015). Increased inbreeding can result, at minimum, in a further reduction of genetic diversity as rare, possibly beneficial alleles maintained in heterozygotes are lost due to genetic drift (Grueber et al. 2015). Inbreeding can also result in inbreeding depression, as deleterious, recessive alleles are more likely to be homozygous and expressed (Freeland et al. 2011).

Finally, increased homozygosity and overall loss of genetic diversity can be dangerous because individuals in the populations are so genetically similar that they are much more likely to all be negatively affected by a single parasite or pathogen. For example, Raymond et al. (2016) showed that populations of *C. elegans* that were more outbred, with higher genetic diversity, had higher fitness in the presence of a parasite compared to an inbred line with lower genetic diversity. The outbred lines were better able to adapt to the parasite than the inbred lines (Raymond et al. 2016).

2.2 Habitat Fragmentation and Amphibians

Amphibians possess several characteristics that make them more vulnerable to habitat fragmentation and destruction than other taxonomic groups (e.g., Silva et al. 2003; Marianna et al. 2009). Physiologically, amphibians are ectotherms with permeable skin that must be kept moist, and thus they are not tolerant of dry and hot environments (AmphibiaWeb 2017). Highly

fragmented habitats contain a larger proportion of edge habitat and less core habitat than an undisturbed area, and edge habitat tends to be hotter and drier than the core or preceding habitat, particularly for forest-dwelling species (Silva et al. 2003). Lannoo et al. (1994) documented reductions in both abundance and species richness of amphibians that inhabited a fragmented forest in Iowa, compared to that same forest 70 years prior when it was still a continuous habitat. Species abundance decreased by three orders of magnitude over the 70 years of increasing fragmentation, suggesting that fragmentation was responsible for the decline in species abundance (Lannoo et al. 1994). Amphibians are also more susceptible to habitat fragmentation compared to other vertebrates due to their small body size and restricted mobility (Silva et al. 2003). These factors reduce the dispersal abilities of amphibians and put them at a further disadvantage when habitat fragmentation makes dispersal more difficult (Krug and Prohl 2013).

Salamanders are a group of amphibians that is widely distributed in North, Central, and South America, Europe and some of eastern Asia (Petranka 1998, Davic and Welsh 2004). Depending on the species, their habitat could include leaf litter, grasslands, underground retreats, caves, streams, ponds, vernal pools, and a variety of other forests elements (e.g., tree holes; Petranka 1998). This study focuses on the terrestrial woodland salamanders in the genus *Plethodon,* which are thought to play a critical role in altering biotic and abiotic environments (Davic and Welsh 2004). Salamanders are considered biotic regulators of ecosystems as they contribute to ecosystem stability through predation on vertebrates (other salamanders) and invertebrates (Davic and Welsh 2004). Davic and Welsh (2004) suggest them for use as ecological indicator species to determine the health of the habitat they reside in due to their interactions with many other species and their habitat requirements. In addition to playing a crucial role in food webs as both predator and prey to other species, woodland salamanders also

have strict soil/air humidity, temperature, and soil pH requirements which must be met for them to thrive in the forest. Davic and Welsh (2004) suggest that monitoring of salamander populations can help determine the overall health of ecosystems. Salamanders are often used in studies that develop concepts for biodiversity conservation, multispecies planning, and the management of wildlife habitat relationships (Johnson and O'Neil 2001). In this study I use a common salamander species to develop an understanding of how its genetics are affected by habitat fragmentation. Studies like mine may help guide conservation efforts for more threatened and endangered amphibian species, as fragmentation may have similar effects on these species.

2.3 *Plethodon cinereus* study system

There are currently 190 known salamander species in the United States and 66 (~35%) of those are considered near threatened, vulnerable, or endangered, while one species is listed as extinct (AmphibiaWeb 2017). Of these 190, 146 (~77%) belong to the family of lungless salamanders, Plethodontidae (AmphibiaWeb 2017). Globally, the plethodontids are the largest family of salamanders (458/695 species, ~66%; AmphibiaWeb 2017), and understanding the risks they face with respect to habitat loss and fragmentation is critical to conserving global salamander diversity. The eastern red-backed salamander (*Plethodon cinereus*) may be useful as a model organism to better understand how fragmentation affects amphibian population genetics, due to its extremely high abundance (Petranka 1998) and because it is closely related to several threatened, North American plethodontids (e.g., *Plethodon shenandoah*; Fisher-Reid and Wiens 2011). *Plethodon cinereus* is a terrestrial, woodland salamander found in northeastern North America (Petranka 1998). *P. cinereus* prefer mature forests with an abundance of leaf litter and cover objects (e.g., rocks, logs) for them to hide under during the day (Petranka 1998).

In some forests the population density of the *P. cinereus* is quite high (> 2 individuals per m^2 in Virginia; Petranka 1998), which helps these populations maintain high levels of gene flow and genetic variation. Previous mark and recapture and genetic studies have suggested *P. cinereus* individuals do not disperse very far or often (e.g., Cabe et al. 2007), but that they are capable of moving greater distances if new forest habitat opens up (e.g., Marsh et al. 2004a) or to return to their territory after being displaced (e.g., Marsh et al. 2007; Marsh et al. 2008), provided there are minimal dispersal barriers. However, gene flow between *P. cinereus* populations is clearly impacted by both natural (e.g., streams; Marsh et al. 2007) and man-made dispersal barriers (e.g., roads; Marsh et al. 2004b; 2008). If these barriers are strong enough to cause habitat fragmentation, we may see increased genetic differentiation and, eventually, the other problems discussed previously.

2.4 Gibbs's Study of Fragmentation and Population Genetics

Gibbs (1998) attempted to address how habitat fragmentation might influence population genetics when molecular ecological tools were first becoming popular. Gibbs (1998) examined the patterns of genetic differentiation in *P. cinereus* populations in Connecticut forests of different sizes. He categorized 17 subpopulations within two geographic areas based on size and land use history. Six forest sites in Norfolk, Connecticut were categorized as "unfragmented." These sites were all part of a single undisturbed forest (Great Mountain Forest, >1000 ha in area) that had been in place since the 1600s and had no anthropogenic barriers. Eleven sites in Milford, Connecticut were categorized as "fragmented" with sizes ranging from 0.3 – 224 ha. Most were recovering secondary forest that had been completely converted to pasture in the mid 1700s

(Gibbs 1998). Gibbs' study found that genetic diversity within subpopulations was not altered, but did discover enhanced genetic differentiation between the subpopulations.

In the 18 years between Gibbs (1998) and the present study, molecular ecology and methods to explore population genetics have progressed rapidly with faster, cheaper, and more reliable techniques (Freeland et al. 2011). Thus, studies like Gibbs (1998) can be reassessed and confirmed with today's technological advances for less money and with much greater confidence in their results. In particular, Gibbs (1998) used RAPD (random amplified polymorphic DNA) markers. These markers use randomly generated probes to isolate and amplify DNA fragments, creating a genetic "fingerprint" represented as a unique banding pattern on a gel (Freeland et al. 2011). Although this technique is effective at uncovering genetic diversity, it is extremely sensitive to contamination from other species (probes are not species-specific) and RAPD assays are difficult to replicate due to the randomness of the amplification process (Freeland et al. 2011). RAPD assays are also dominant markers, masking the presence of heterozygotes, and thus underestimating genetic variation (Freeland et al. 2011). While RAPD was one of the better methods available during the 1990's, advances in both molecular and computational technology have provided us with superior ways to measure genetic variation (Freeland et al. 2011).

My study revisits Gibbs (1998) with these newer genetic techniques. These newer methods will provide clearer insight to the genetic effects of habitat fragmentation and possibly catch genetic changes masked by RAPD assays. I used species-specific, co-dominant microsatellite loci (Connors and Cabe 2003; Fisher-Reid et al. 2013), developed specifically for *P. cinereus*, greatly reducing the risk of contamination (Freeland et al. 2011). Microsatellites are co-dominant, which allows me to see all gene combinations for a complete picture of genetic variation and differentiation in my sample populations (Freeland et al. 2011). I visited local

forests in Massachusetts for the collection of genetic material, and like Gibbs (1998), I included fragmented and continuous forests based on the size of forest uninterrupted by anthropogenic barriers (e.g., roads, urban areas). I then analyzed each population with metrics of genetic diversity and variation to explore the effects of fragmentation on the population genetics of *P. cinereus.*

3. Methods

3.1 Field Sites

I selected four forests within Massachusetts with different levels of fragmentation. Fragmentation was estimated by the total area of the forest that was not divided by roads, agricultural fields, or other human development. Smaller forest areas were considered to be highly fragmented, while larger forest areas were considered to be more continuous. Fragmented sites included Great Hill Forest, on the campus of Bridgewater State University in Bridgewater, MA (BSU), and Pine Knoll (BCAHS 01) and Oak Grove (BCAHS 02) forests at Bristol County Agricultural High School in Dighton, MA. For continuous sites, I sampled on private property (with permission of the landowner) that formed a continuous forest with Savoy Mountain State Forest in Florida, MA (SSF) and at three sites at Mt. Greylock State Reservation, in Adams, MA (MTGL01–03). I determined the approximate areas of the fragmented sites by drawing an area-estimating polygon around each site in Google Earth (Google Earth 2017). Continuous sites were all >1000 ha, while fragmented sites were < 60 ha.

3.2 Data Collection

Tissue collection was approved by Bridgewater State University's Institutional Animal Care and Use Committee (IACUC case number 2015-1) and conducted under scientific collecting permit #024.16SCRA issued to M. C. Fisher-Reid by Massachusetts Department of Fisheries and Wildlife (with M. Tucker as a subpermitee). *Plethodon cinereus* tail clips were collected from 20-30 individuals from each location by pinching the last 1 cm of tail with forceps, making use of the salamanders' natural response to detach their tail when attacked by predators (e.g., Cabe et al. 2007). Tail clips were preserved in 95% ethanol and stored at –80ºC. DNA was extracted using a Qiagen® DNeasy® Blood and Tissue kit following the quick start tissue extraction instructions. Several microsatellite markers have been developed for *P. cinereus* (e.g., Connors and Cabe 2003; Fisher-Reid et al. 2013), and I tested several of these for amplification success. Of the 13 available markers, seven markers amplified well in a subset of individuals from my populations, and I proceeded to amplify these seven loci (Table 1) in the remaining individuals using polymerase chain reaction (PCR; conditions in Table 1). All microsatellite loci used one of two standard touchdown (TD) programs during PCR. Microsatellite loci MS3241, MS3544, MS1718, and MS4953 used TD50. Microsatellite loci PcJX24, PcII14, and PcA18 used TD 55. The difference between these programs is their starting and finishing annealing temperatures: TD50 starts at 55ºC and declines in 0.5ºC increments to 50ºC over the first 10 cycles, followed by 25 cycles at 50ºC. TD55 starts at 60ºC and declines in 0.5ºC increments to 55ºC over the first 10 cycles, followed by 25 cycles at 55ºC. PCR products were checked using gel electrophoresis, and clean products were sent to either Eurofins Genomics or Boston Children's Hospital for fragment analysis. Results were scored manually

using Geneious 8.1. After scoring, fragments were manually checked for stutters and refined in R using the 'MSAT Allele' package (Alberto 2013).

3.3 Data Analysis

I first compared the overall genetic diversity in continuous and fragmented populations using Genepop on the Web (Raymond and Rousset 1995; Rousset 2008) and FSTAT (Goudet 1995) to examine expected and observed heterozygosity, allelic richness, and level of inbreeding (as measured by Wier and Cockerham's F_{IS}). Since some genetic differentiation can be attributed to isolation-by-distance (Freeland et al. 2011), and my sites varied from <1 km to more than 200 km apart, I also tested for isolation-by-distance using Genepop on the Web. In this analysis, I expected there to be strong isolation-by-distance between western MA (SSF, MTGL) and eastern MA sites (BSU, BCAHS), and looked for pairs of sites that showed more isolation than expected given their distance (i.e., above the regression line).

I used STRUCTURE (Pritchard et al. 2000) to determine how genetically distinct each subpopulation was. STRUCTURE determines the most likely number of genetic clusters, *K*, and assigns individuals to each cluster based on their genetic profiles. STRUCTURE was run for K = 1 – 10 with five iterations per *K*. Based on previous STRUCTURE analyses of *P. cinereus* microsatellite data (Fisher-Reid et al. 2013), I set the burnin period to 100,000 followed by 2 x 10^6 MCMC reps post burnin. Following the justification laid out in Fisher-Reid et al. (2013), I used the 'no admixture' model with location priors turned on, and allele frequencies correlated. STRUCTURE results were analyzed using both the ΔK method (Evanno et al. 2005) as a measure of primary or top-level structure and log likelihood scores (Pritchard et al. 2000) as a measure of secondary structure in the program Structure Harvester (Earl and VonHoldt 2012).

4. Results

All analyses were done twice: once with the SSF population, and once without the SSF population. The data from the SSF population were suspiciously uniform across individuals (nearly identical fragment analysis patterns), and two loci (PcII14 and PcA18) did not produce clean fragment peaks at all, despite clean PCR gels, and thus were left out of analyses as missing data. These issues, combined with the results when the SSF population was included (Figs. 3 and 4), display SSF as a clearly distinct, separate, and genetically isolated from all other populations, even MTGL populations that were < 15 km away, led me to be mistrustful of these data overall. The results I present and discuss below focus on those analyses that excluded SSF; however, the analyses that include SSF are presented in Table 2 and Figures 3 and 4, and are briefly discussed in the Discussion.

Mean allelic richness for six subpopulations (all except SSF) at all seven markers was 3.86 (95% CI 3.46 – 4.26; Table 2). The fragmented populations (BSU, BCAHS 01–02) generally had lower allelic richness than the continuous populations (MTGL01–03), and this result was significant for BCAHS01 and BSU. Mean allelic richness in the fragmented populations was significantly lower than mean allelic richness for all populations (Table 2). Continuous population MTGL02 had a significantly greater mean allelic richness than the mean allelic richness for all populations (Table 2). Four of the subpopulations deviated from Hardy-Weinberg Equilibrium (HWE) due to an excess of homozygotes (significant, positive F_{IS} values; Table 2) and one from an excess of heterozygotes (significant, negative F_{IS} values; Table 2). BCAHS02 was in HWE (Table 1).

The isolation-by-distance (IBD) results are approaching significance with variation in genetic isolation within "close" and "far" pairs of populations (IBD slope = 4.59×10^{-4}, Mantel test $r = 0.646$, $P = 0.074$; Fig. 1). Over short distances, the fragmented populations show stronger isolation values than the non-fragmented sites (circled points in Fig. 1), however, more data would be necessary to be confident of the regression line on which this result depends.

STRUCTURE analysis recovered two clear geographic clusters, eastern and western MA, as the top level of population structure ($K = 2$, $\Delta K = 361.42$ $Ln\ P(K) = -1374.62$; Fig. 2). Secondary structure also occurs between BCAHS and BSU habitats, while MTGL subpopulations are well mixed. ($K = 4$, $\Delta K = 14.93$ $Ln\ P(K) = -1262.58$; Fig. 2).

5. Discussion

This study aimed to test the hypotheses that increased urbanization and fragmentation of forest habitats will have negative effects on the genetic variation and increase genetic structure of amphibian species (e.g., Gibbs 1998; Marianna et al. 2009; Silva et al. 2003). It is also a follow up of Gibbs (1998) using modern molecular tools. My results give partial support for the hypothesis that there will be a greater amount of genetic diversity in continuous habitats compared to fragmented habitats (Table 2). All three continuous sites (MTGL01-03) had higher allelic richness and two of these had lower F_{IS} values (MTGL02-03) compared to the other sites (Table 2). Both of these measures indicate higher genetic diversity compared to the other sites, and these results suggest individuals may be better able to easily disperse between subpopulations (e.g., Marsh et al. 2007; Cabe et al. 2007).

Isolation-by-distance and STRUCTURE results partially support the hypothesis that the fragmented habitats have increased genetic differentiation at short distances. In the isolation-by-

distance plot, pairs of fragmented habitats (BSU vs. BCAHS01 and BSU vs. BCAHS02; circled in red; Fig. 1) were more genetically distinct than expected for the geographic distance between them (above a regression line; Fig. 1) compared to the continuous habitat points. Because there are only two distance classes included (near and far, indicated by two point clouds in Fig. 1), additional data from intermediate distances are needed to truly determine how far above the regression line circled fragmented sites are (i.e., whether the differentiation observed is significant). BSU and BCAHS are more genetically distinct from each other (Figure 1) than any of the MTGL populations are from each other (a result further supported by the STRUCTURE analysis, see below). This result may be indicative of inbreeding and a lack of migration occurring between these subpopulations (e.g., Andersen et al. 2004; Arens et al. 2007).

Similarly, the STRUCTURE analysis revealed primary structure of two distinct subpopulations ($K = 2$; Fig. 2), which is not surprising due to the distance between our eastern and western sampling areas, as the sites were so far apart the individuals would never meet to exchange genetic material. When examining the STRUCTURE results further for secondary structure, the fragmented habitat populations split into distinct subpopulations that corresponded with their geographic location (i.e., BSU and BCAHS were distinct; $K = 4$ Fig. 2), while the MTGL continuous forest populations were much more genetically mixed (Fig. 2). This is what was expected given previous studies which show that amphibians have trouble dispersing across a single road alone, due to their size (Marsh et al. 2008; Marianna et al. 2009) and there is a large degree of urbanization and development between the BCAHS and BSU sites, so it would be expected that their populations would be more strongly genetically differentiated. Overall, these results support my hypothesis that increased fragmentation will lead to reduced genetic diversity (Table 2) and increased population structure (Figures 1 and 2).

As described in the Results above, the fragment analysis of SSF data was suspicious due to three reasons. The first reason was that many individuals displayed identical patterns and similarly sized peaks. A second reason was excessive amounts of stuttering within the fragment analysis calling the legitimacy of the observed peaks into question. The third reason was that several individuals displayed three strong peaks when we would only expect at most two peaks for heterozygotes or one peak for homozygotes due to *P. cinereus* being a diploid species. The uniqueness of these fragment abnormalities also appears when they are included in my analyses. In the isolation-by-distance plot, SSF strongly diverged from all other populations, no matter the distance (circled points in Fig. 3). In the STRUCTURE analysis that includes SSF, top level structure recovered three distinct subpopulations: the eastern fragmented forests making one cluster, the western continuous MTGL subpopulations and the western continuous Savoy State Forest making their own distinct clusters (K = 3; Fig. 4). When this STRUCTURE analysis was analyzed further for secondary structure, the BSU and BCAHS forest populations became distinct from one another, as before, the MTGL populations became more genetically mixed, as before, and SSF continued to be one distinct genetic cluster (K = 5; Fig. 4). In addition to these curious results, the raw fragment data had extremely messy peaks, making it hard to determine different alleles, and several individuals had completely identical fragment profiles at some loci (unlikely, given the size of *P. cinereus* populations and number of individuals sampled). These difficulties were only encountered with the SSF data, leading to the conclusion that re-analyzing these data to confirm the validity of the data would be beneficial.

If the SSF data are redone and the results obtained match the current results, then the cause could be because MTGL and SSF, while close together, are atop two different mountain peaks. Janzen (1967) documented the ability of mountains to prevent movements of animals and

plants, and observed greater species isolation between mountains. Janzen hypothesized that this was due to the valley between the mountains acting as a natural dispersal barrier, as species upon the mountain are less likely to migrate down a mountain and up another, being more inclined to stay on the current mountain, effectively becoming an isolated population (Janzen 1967). While Janzen's study was conducted in tropical mountain passes, where the effect might be stronger due to more stable annual climates, this effect could be observed in non-tropical montane habitats as well. If this effect were occurring in population, data from continuous forests not separated by varying elevations would be needed, e.g., along a continuous ridgeline, to further help explain the present occurrence.

6. Future Work

There are several ways with which to expand this project to improve our confidence in the results. First, additional collection and analysis of DNA samples from intermediate forest sizes and distances will help confirm the isolation-by-distance and STRUCTURE results. I was unable to re-amplify the SSF data due to time constraints, and given the suspicious nature of results that include SSF, these data should be completely redone. The addition of the SSF data and of moderately fragmented, intermediate distance sites to fill in the space lacking data points in the isolation-by-distance figure, would help give a better picture of how habitat fragmentation affects the genetics of *P. cinereus* populations.

7. Acknowledgements

This project was funded by the Office of Undergraduate Research at Bridgewater State University through an Adrian Tinsley Program (ATP) Summer Grant to Nathan Pierce and Matthew Tucker, and a Fall 2016 ATP Semester Grant to Matthew Tucker. I would like to thank Nathan Pierce for help with field and lab work, and Anna Back, Brian Bastarache, Mariah Fossella, Jonathan Roling, and the students at Bristol County Agricultural High School for their help conducting fieldwork. Thanks to Dr. Bloch and Dr. Roling for being my thesis committee members. Additional thanks to Dr. Roling for housing and feeding us during our stay in western MA, and for permission to collect samples on his property. Special thanks to my thesis director, Dr. M. Caitlin Fisher-Reid, for expanding my knowledge on population genetics and helping me through the process of becoming a proper researcher.

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9. Figures and Tables

Table 1: Primers for Microsatellite Loci

Microsatellite loci with their respected primer sequences, optimal annealing temperature, targeted repeat motif, and their literary source. Each locus has a fluorescently dyed primer indicated by the bracketed label (e.g.,[5HEX]) at the 5' end. Primer sequences are followed by the concentration they were used at during PCR.

Microsatellite Locus	Primer Sequence (5' → 3') [Concentration]	Repeat Motif	PCR Annealing Temperature	Source
MS3241	F: [5HEX]GGTCCCCTTTGTATCTACTTTG [10 μM]	CATT	55 -> 50	Fisher-Reid et al. 2013
	R: TGCCGCCTAATTGCATTCC [6 μM]			
MS3544	F: [6FAM]GCACAAAGCAAAGAGGAGAAA [10 μM]	CATT	55 -> 50	Fisher-Reid et al. 2013
	R: TCATTCATGTCTGAACTAGTGTG [6 μM]			
MS1718	F: ATTCGGCATATTTTTCACTCTAA [6 μM]	CATT	55 -> 50	Fisher-Reid et al. 2013
	R: [6FAM]GCAGCAGGTAGGTCTATCACG [10 μM]			
MS4953	F: TGTGCGTCCTAAAAGCCATC [6 μM]	CAA	55 -> 50	Fisher-Reid et al. 2013
	R: [5HEX]TCACCTAGCTCACTTGCCC [10 μM]			
PcJX24	F: [HEX]GCTGCTGCTAAGCACTCCTC [10 μM]	i(AC)33	60 ->55	Connors and Cabe 2003
	R: ATCTCCTCCAACCGATTTCC [6 μM]			
PcII14	F: AACCCACACCAGATCCACTC [6 μM]	i(AC)23	60 ->55	Connors and Cabe 2003
	R: [TET]TGGTTTGCTGTCTTCTTGC [10 μM]			
PcA18	F: [FAM]CAACTCCCACTTCCAGAAC [10 μM]	i(AC)35	60 ->55	Connors and Cabe 2003
	R: GAGGGGAGAAGTGTTGTAACG [6 μM]			

Table 2: Genetic summary of each population.

Diversity estimates (allelic richness, heterozygosity expected (H_E), heterozygosity observed (H_O), and F_{IS}) are averaged for each population across seven microsatellite loci (except for SSF, which only had five loci amplified). Bolded values of allelic richness are outside the 95% confidence intervals for the mean from all subpopulations (when SSF is not included: mean = 3.86; 95% CI = 3.46 – 4.26; when SSF is included: mean = 4.05; 95% CI = 3.49 – 4.61). Bolded values of F_{IS} are significant at $P < 0.05$.

Site	Location	Coordinates	Area (ha)	N	Mean Allelic Richness	Mean H_E	Mean H_O	F_{IS}
BCAHS01	Dighton, MA	41° 49' 55.2714" -71° 6' 55.7994"	4.2	13	**3.210**	0.58	0.4918	**0.199**
BCAHS02	Dighton, MA	41° 50' 27.348" -71° 6' 48.5634"	12	6	3.894	0.6414	0.5667	0.0995
BSU	Bridgewater, MA	41° 59' 13.1784" -70° 57' 29.96"	52.2	24	**3.311**	0.6020	0.5083	**0.205**
MTGL01	Adams, MA	42° 35' 55.791" -73° 11' 59.2002"	>1000	9	4.181	0.7286	0.5388	**0.2467**
MTGL02	Adams, MA	42° 35' 33.2082" -73° 11' 54.8406"	>1000	6	**4.656**	0.7622	0.7476	**-0.0210**
MTGL03	Adams, MA	42° 35' 32.4204" -73° 12' 4.8702"	>1000	9	3.914	0.6966	0.6687	**0.0447**
SSF	Florida, MA	42° 41' 07.1" -73° 03' 37.2"	>1000	24	**5.167**	0.6493	0.6847	**0.0511**

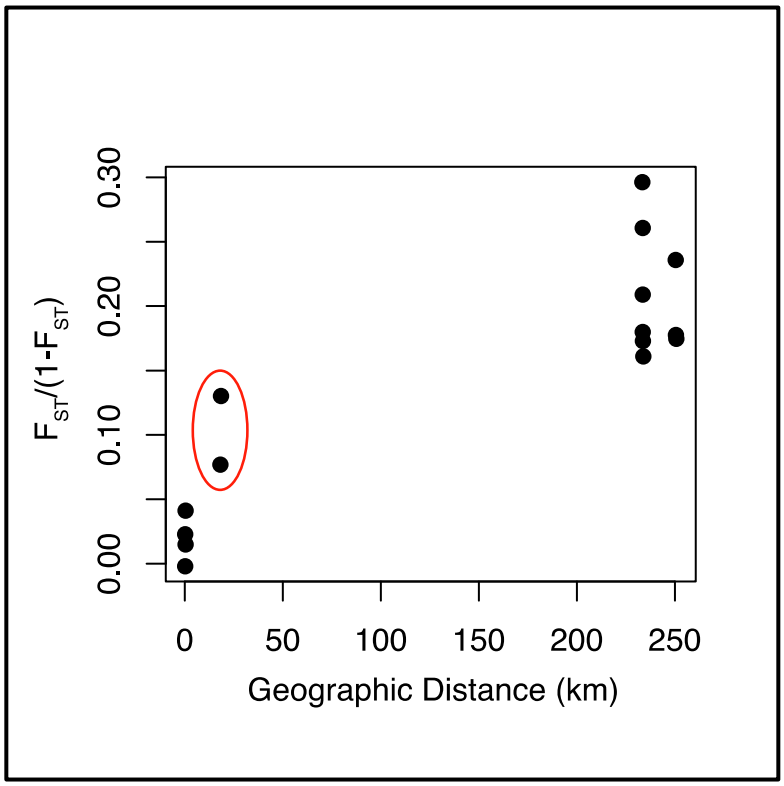

Figure 1: Genetic isolation-by-distance (IBD) without Population SSF.

Each point represents a pairwise comparison between two of the six subpopulations (IBD slope = 4.59×10^{-4}, Mantel test $r = 0.646$, $P = 0.074$). As expected, subpopulations that are closer together exhibit less genetic isolation than those that are far apart, however, within the "close" and "far" clusters, there is a lot of variation. Points circled in red are BSU vs. BCAHS01 and BCAHS02 populations, which show stronger isolation than BCAHS01 vs. BCAHS02 and each pair of MTGL populations, suggesting that fragmentation does lead to greater isolation over shorter distances.

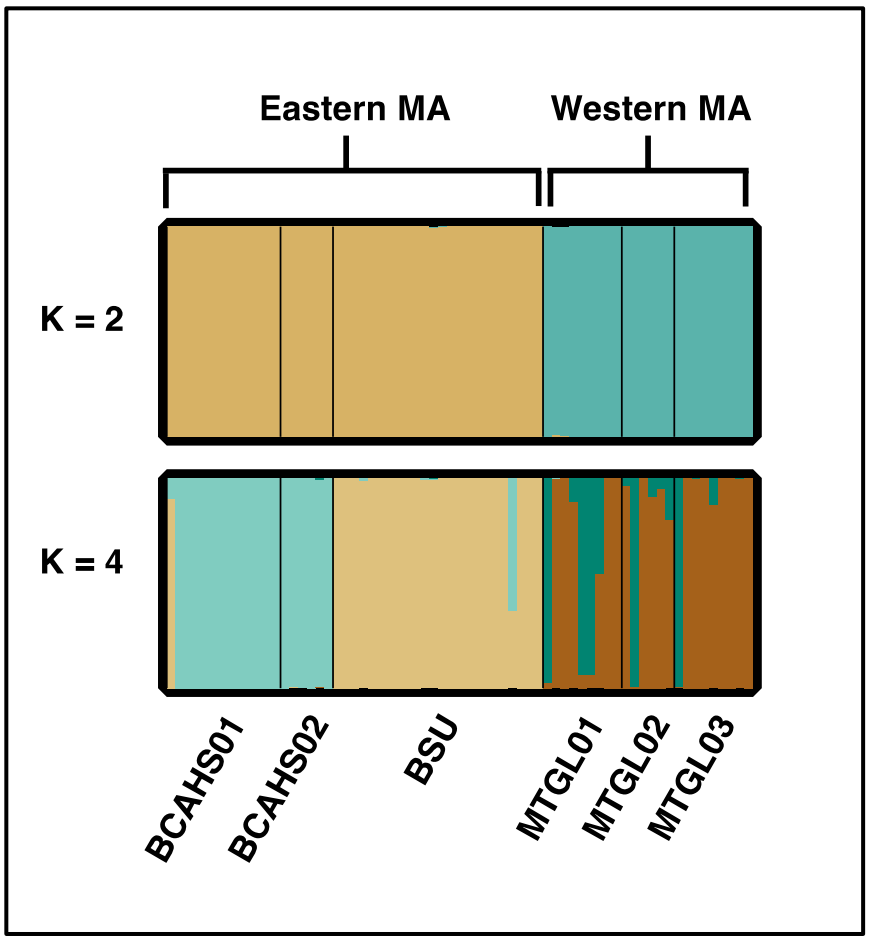

Figure 2: STRUCTURE results without Population SSF.

Each thin black line separates a sample population, each color is a genetic cluster, and each individual is a single colored line. The top graph (K = 2, ΔK = 361.42, *Ln P(K)* = -1374.62) represents the top level of population structure (selected using the ΔK method; Evanno et al. 2005), clearly distinguishing between eastern and western Massachusetts. The bottom graph (K = 4, ΔK = 14.93 *Ln P(K)* = -1262.58) and represents the secondary structure (selected using the best log likelihood score; Pritchard et al. 2000), showing clear separation between BSU and BCAHS, and that MTGL populations are well mixed, genetically.

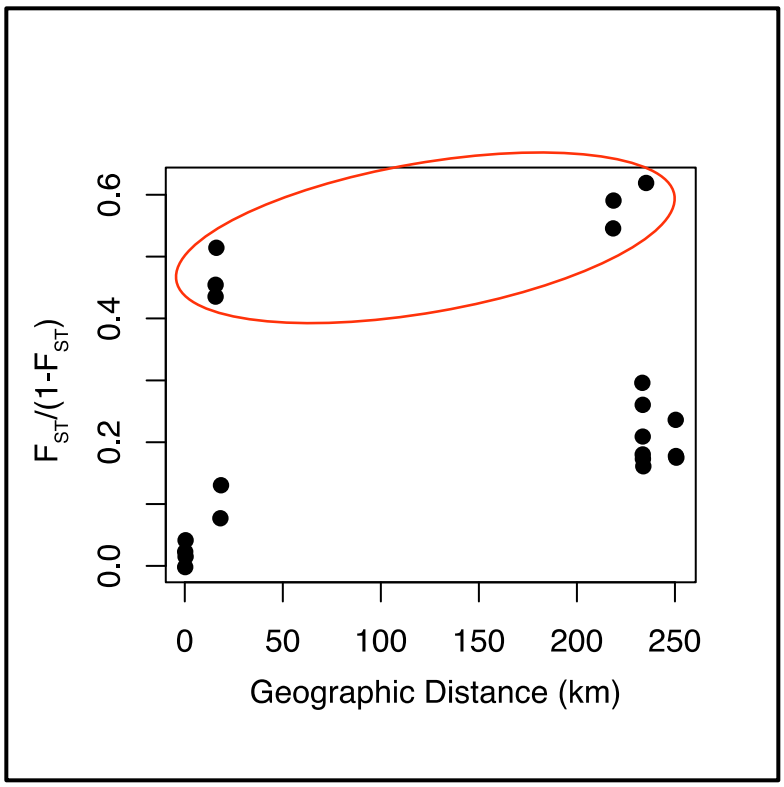

Figure 3: Genetic isolation-by-distance (IBD) with Population SSF.

Each point represents a pairwise comparison between two of the seven subpopulations (IBD slope = 4.9133 x 10^{-4}, Mantel test r = 0.3098, P = 0.1322). Subpopulations that are closer together exhibit less genetic isolation than those that are far apart. The non-SSF pairs are largely unchanged from Figure 1. The points circled in red are pairs that include the SSF population, demonstrating that it is distinctly different from all locations, regardless of distance (high isolation values at near and far distances). This result cast doubt on the reliability of the SSF data, and genetic data collection should be repeated for confirmation.

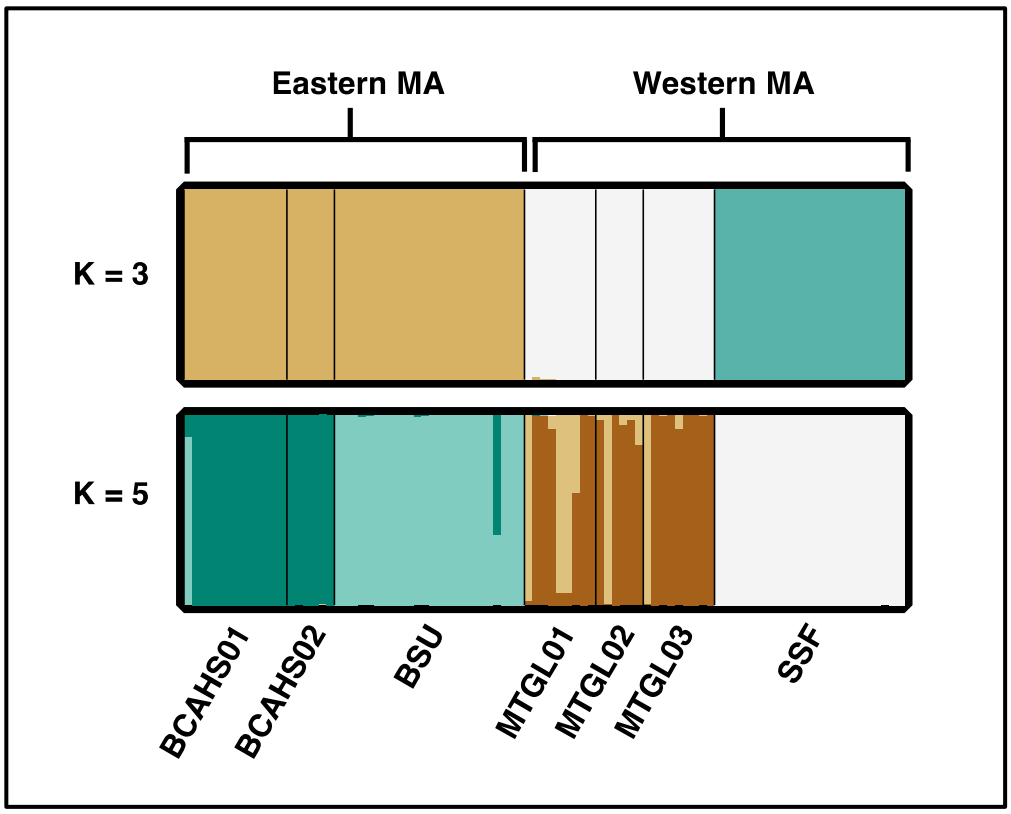

Figure 4: STRUCTURE results with Population SSF.

Each thin black line separates a sample population, each color is a genetic cluster, and each individual is a single colored line. The top graph, $K = 3$ ($\Delta K = 159.023$ *Ln P(K)* = -1650.44), represents the top level of population structure (selected using the ΔK method), clearly distinguishing between eastern and western Massachusetts. The bottom graph, $K = 5$(ΔK = 6.484 *Ln P(K)* = -1534.2), and represents the secondary structure (selected using the best log likelihood score) The results for non-SSF populations are identical to Figure 2, however, SSF comes out as its own unique cluster in both plots here.