CONSERVATION GENETICS OF A SPOTTED SALAMANDER (*AMBYSTOMA*

MACULATUM (SHAW 1802)) LOCAL POPULATION IN SOUTHEAST

TENNESSEE

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ABSTRACT

Currently there is a worldwide amphibian decline with numerous causes. Because of this, it is important to understand how genetic variation in local amphibian populations is affected by disturbance. The goal of this study was to assess the genetic impacts of past chemical and ammunition storage and present industrialization on a local population of *Ambystoma maculatum* (Shaw 1802) using thirteen microsatellite markers. Most loci exhibited no loss of heterozygosity but the low effective population size may indicate vulnerability to future declines. No population substructure or sex-biased dispersal was detected. Possible explanations for the reduction in genetic diversity at four loci include a past anthropogenic decline, natural population cycles, and impending effects of a decline. Close monitoring of the population is necessary because genetic diversity may decline in the future due to current anthropogenic disturbances.

Keywords: Ambystoma maculatum, spotted salamander, conservation genetics, population, Tennessee

ACKNOWLEDGEMENTS

project and tolerated "I can't do that right now I'm working" innumerable times. I'm sure I've left someone out and thank anyone I did.

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LIST OF ABBREVIATIONS

SVL, snout-to-vent length

MVP, minimum viable population size

SBD, sex-biased dispersal

ES, Enterprise South

N, north division

W, west division

S, south division

E, east division

PCR, polymerase chain reaction

bp, base pairs

Na, number of alleles

AR, allelic richness

SE, standard error

HWE, Hardy-Weinberg equilibrium

MCMC, Markov Chain Monte Carlo

IAM, infinite allele model

SMM, stepwise mutation model

TPM, two-phase model

LIST OF SYMBOLS

H_e, expected heterozygosity

H_o, observed heterozygosity or null hypothesis

F_{IS}, fixation index

N_e, effective population size

K, number of genetic clusters

X, observed set of allele frequencies

CHAPTER 1

INTRODUCTION

Literature Review

Amphibians are generally less vagile than members of other vertebrate taxa (Blaustein et al. 1994). Genetic structure at small geographical scales is often apparent as a result of population clustering within favorable habitats (Jehle et al. 2005). It is possible that amphibian populations are less genetically diverse than populations of more vagile taxa although this has yet to be explored (but see Selander and Kaufman 1973). If this is the case, amphibians may be more vulnerable than other vertebrate taxa to negative population genetic effects of habitat alteration and extinction risks may be greater (Noel et al. 2007; Todd et al. 2009). Conservation genetic studies on amphibians reveal that genetic diversities are frequently lower in populations situated in areas with high levels of habitat alteration as opposed to those in areas with lower levels of disturbance (Hitchings and Beebee 1997; Keller and Largiader 2003; Shaffer et al. 2000; Templeton et al. 1990; Young et al. 1996).

Genetic factors play important yet historically underappreciated roles in population declines and extinction (Frankham 1995a). Maintenance of adequate genetic diversity is necessary for population persistence because sufficient genetic diversity helps to ensure adaptability to changing environmental conditions and helps to protect against homozygosity for harmful alleles (Hedrick and Miller 1992; Lande 1988; O'Brien 1994). Even at neutral loci not subject to natural selection, genetic diversity may correlate positively with fitness because forces

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that reduce genetic variability (e.g., genetic drift) are likely to operate simultaneously at many loci across the genome (Hedrick 2001). Neutral loci may also be linked to loci subject to natural selection. Genetic variation at these loci may correspond to that of loci under selection (Barton 2000). Deleterious effects that may result from population decline include reduced gene flow due to reduced probability of receiving migrants, increased magnitude of random genetic drift owing to smaller population size, increased probability of expression or fixation of harmful mutations, and decreased genetic variability on which natural selection can act (Burger and Lynch 1995; Lande 1994).

Forces Affecting Genetic Variability

Gene Flow

Gene flow is important for maintaining the genetic variability of amphibian populations because it results in the introduction of new alleles and helps to counteract the effects of drift. Gene flow refers to the exchange of alleles between populations as a result of interbreeding between members of different populations (Wright 1931). Gene flow between genetically differentiated populations typically results in linkage disequilibrium between loci. Linkage disequilibrium is the non-random association of genotypes at two or more loci because of low recombination frequencies. As the name suggests, it may result because loci are physically linked. However, linkage disequilibrium may also stem from two or more loci having different allele frequency profiles in two or more populations coming into contact with each other. Divergent allele frequencies between two or more genes typically cause them to recombine less frequently (Hartl and Clark 2007). Therefore, it can serve as an indication as to whether genetically differentiated populations are situated close to a population under study.

Patterns of gene flow in amphibians tend toward increasing isolation with less landscape permeability or increasing anthropogenic habitat alteration (Chan and Zamudio 2009; Funk et al. 2005; Giordano et al. 2007; Greenwald et al. 2009a; Greenwald et al. 2009b; Sandberger et al. 2010; Spear et al. 2005; Squire and Newman 2002; Stevens et al. 2006; Zhan et al. 2009). Many amphibian species exhibit high breeding site fidelity, which may also reduce gene flow (Gamble et al. 2007; Huste et al. 2006). Isolated populations may be more prone to extinction than connected populations because they are often derived from small numbers of founders and may be subject to founder effects (Frankham and Ralls 1998).

Structure of Amphibian Populations

Pond-breeding amphibians frequently behave as metapopulations composed of smaller demes as functional units (Marsh and Trenham 2001; Smith and Green 2005). This fact has important conservation implications as loss of dispersal routes between local populations increases isolation and may result in subsequent loss of genetic diversity. Information on genetic clustering within local amphibian populations is virtually absent from the literature but is theoretically possible because of polygamous mating systems (Tennessen and Zamudio 2003), habitat heterogeneity (which is the case in this study), or immigration. Yet such information is crucial for amphibians because it can help to provide a greater understanding of mating systems, gene flow, and differential selective pressures. In the present study, a local population is defined as a breeding aggregate of *Ambystoma maculatum* occupying a single vernal pool. This follows the definition of Waples and Gaggiotti (2006). The term "population" is used interchangeably with local population unless otherwise indicated.

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Random Genetic Drift

Random genetic drift occurs naturally in all population genetic systems because of finite population sizes but is of concern when populations are small in size or when drift is strong enough to override counteracting forces such as gene flow. Random genetic drift refers to random changes in allele frequencies resulting from sampling error of the gametic pool of a population as only a small number of the total gametes produced by any one generation become the next generation (Hartl and Clark 2007; Kimura 1955). One allele drifts toward fixation (complete homozygosity) and all others will be lost if counteracting forces are not strong enough to override genetic drift. When populations are smaller, the magnitude and effects of random genetic drift are increased because there is typically less genetic diversity and increased sampling error of gametes contributing to the next generation (Kimura and Ohta 1969; Kimura 1955). Harmful mutations may become fixed in a small population by chance alone (Glemin et al. 2003; Kimura et al. 1963; Whitlock and Bourguet 2000). The role of genetic drift in shaping genetic variation in amphibian populations is not well studied. Drift may be a predominant force in amphibian populations with a history of anthropogenic disturbance (Johansson et al. 2007; Lesbarrères et al. 2006; Wilson et al. 2008), but it is possible that sufficient gene flow can override its effects in such populations (Jordan et al. 2009).

Population Bottleneck

A bottleneck is a drastic reduction in effective population size that results in only a subset of former genetic variation in a population being maintained. Although a bottleneck results in a reduction of genome-wide diversity, there is typically an excess of heterozygosity relative to that expected at mutation-drift equilibrium because heterozygosity expected at mutation-drift

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equilibrium is proportional to population size (Luikart et al. 1998). Declines in genetic diversity caused by bottlenecks render populations more susceptible to further decline by means of increased inbreeding, leading to even further declines in genetic diversity and even more severe inbreeding and so on by means of what is known as an extinction vortex (Fagan and Holmes 2006; Gilpin and Soulé 1986). Available information suggests that amphibian populations often undergo bottlenecks during periods of high habitat disturbance (Allentoft et al. 2009; Spear et al. 2006).

Effective Population Size

The magnitude of genetic drift and other evolutionary forces (change in allelic frequencies per generation) depends on the effective population size (N_e) and not on the census population size as not all adults contribute to the next generation. N_e is defined as the size of an idealized randomly mating population in which every individual has an equal chance of contributing to the next generation (Charlesworth 2009; Lande and Barrowclough 1987; Wright 1931). Because not all individuals contribute to the next generation in real populations, N_e is usually smaller than the census size and genetic variation is lost at a correspondingly higher rate in response to any evolutionary force that reduces variability (Frankham 2002). N_e is a crucial parameter for conservation genetics but surprisingly few studies have estimated N_e for amphibian populations because of the difficulty in obtaining genetic samples at multiple time points. Available evidence suggests that N_e values for amphibians tend to be small (less than 1,000 individuals) although the primary factors that determine N_e in amphibians are poorly understood (Jehle et al. 2005; Phillipsen et al. 2011; Rowe and Beebee 2004; Schmeller and Merilä 2007; Scribner et al. 2001; Wang et al. 2011). The most direct and powerful methods of estimating N_e

involve taking multiple genetic and demographic samples at different time points at least one generation apart but are often labor intensive and cost-prohibitive (Beebee and Rowe 2004). Single-sample genetic methods, although less powerful, are still useful for estimating N_e because of greater feasibility and a greater number of examples in the literature available for comparison.

An important consideration is to distinguish N_e from the concept of minimum viable population size (MVP). Despite the name, N_e is not the minimum number of individuals required to maintain a population. It is the number of individuals on which genetic changes due to evolutionary forces depend. For example, a population with a large census size but a small N_e tends to rapidly lose genetic variation due to drift in the event of habitat alteration whereas one with both a high census size and high N_e will lose variation more slowly. MVP is the minimum number of individuals required to maintain a population and depends on both genetic and demographic factors (Nunney and Campbell 1993; Soulé 1987).

Inbreeding Depression

Inbreeding depression may result when genetic diversity reaches low levels. Greater levels of inbreeding often correlate with reduced fitness and may result in inbreeding depression, especially in more stressful environments (Bijlsma et al. 2000) such as those found with imperiled amphibian populations. Inbreeding depression probably results from increased homozygosity of harmful mutations (fixation load), leading to reduced fitness (Charlesworth and Charlesworth 1999; Charlesworth and Willis 2009; Wang et al. 1999). The fitness costs of inbreeding can vary depending on environmental conditions and other aspects of a species' mating system. Certain factors, such as a high dispersal rate, can offset any negative effects (Keller 1998). There are too few studies of inbreeding depression in amphibians in order to draw

any broad conclusions. It is possible that inbreeding is a major contributor to extinction in amphibian populations (Rogell et al. 2010; Rowe and Beebee 2003).

Sex-Biased Dispersal

Conservation efforts for amphibian populations can be aided by knowledge of their natural history and ultimate causes of their behavior. Sex-biased dispersal (SBD) is a particularly relevant phenomenon that is important for the conservation of amphibians that migrate to breeding ponds because gene flow may be asymmetrical (the result of one sex dispersing more than the other). Accordingly, genetic variability may be altered if SBD is present. SBD is also a potential cause of genetic heterogeneity within local populations because the dispersing gender is basically composed of two local populations within the same wetland: residents and immigrants (Hartl and Clark 2007; Lampert et al. 2003).

SBD is not well-studied in amphibians although there is extensive information on its proximate and ultimate causes in mammals, birds, and reptiles (Dobson and Jones 1985). Certain examples of studies on sex-biased dispersal in amphibians include *Anaxyrus fowleri* (Fowler's toad), which exhibits evidence of unbiased dispersal (Smith and Green 2006). Hypotheses put forward to explain sex-biased dispersal in birds and mammals are also likely to apply in amphibians. Competition for mates, particularly in polygynous mating systems, is a hypothesis put forward to explain male-biased dispersal (Dobson 1982; Perrin and Mazalov 1999). According to the local mate competition hypothesis, male-biased dispersal will result if competition for females reaches a threshold that causes males to wander in order to search for mates. Resource competition (e.g., for nesting sites) is a hypothesis devised to explain female-biased dispersal (Dobson 1982; Perrin and Mazalov 1999; Perrin and Mazalov 2000).

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Inbreeding avoidance has also been put forth as a mechanism to drive SBD as well, although is unlikely to be the sole reason for sex-biased dispersal because it does not predict which sex will preferentially disperse (Bollinger et al. 1993; Gandon and Rousset 1999; Perrin and Mazalov 1999; Pusey 1987). The existence of SBD implies that one sex is primarily responsible for gene flow and therefore necessitates conservation of both the wetland and surrounding habitat in order to maintain connectivity (Blundell et al. 2002; Fraser et al. 2004; Nehlsen et al. 1991).

The few studies of SBD in amphibians reveal no consistent pattern but male-male competition for females has been suggested for the family Ambystomatidae (Verrell and Krenz 1998) as well as in other salamander families (Denoël and Schabetsberger 2003; Mathis 1991). Competition for resources other than mates has been found to occur among male salamanders (Gabor and Jaeger 1995; Mathis 1990) and may drive males to disperse more than females. Often, sex ratios in the family Ambystomatidae are biased in favor of males (Flageole and Leclair 1992; Williams et al. 2009 but see Homan et al. 2007 and Palis 1997). Earlier arrival of males at breeding sites, which was observed at the study site, may increase breeding success by increasing the number of clutches sired (Tennessen and Zamudio 2003). Parental factors may also play a role in male-biased dispersal as tending to eggs and young may constrain female movement (Revardel et al. 2010).

Summary of Literature Review

The evolutionary forces that are most important in determining local population genetic structures of amphibians are poorly understood (with the exception of gene flow in the context of population connectivity). However, current literature suggests that amphibians may be vulnerable to loss of genetic variation in the face of habitat alteration as lower genetic

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variation is often found in amphibian populations situated in disturbed habitat. Low effective

population sizes may also render amphibian populations highly vulnerable to loss of genetic

variation in the face of habitat alteration. Sex-biased dispersal has not been extensively

investigated in amphibians and available studies show no consistent pattern of results. Together

such findings suggest that more studies dealing with amphibian conservation genetics are

needed.

Background and Research Problem

Currently there is a worldwide amphibian decline with numerous causes including

habitat alteration, invasive species, pollution, commercial exploitation, disease, climate change,

and poor habitat management practices (Alford 2011; Alford and Richards 1999; Collins and

Storfer 2003; Storfer 2003; Stuart et al. 2004). Wetlands may be the most threatened habitats

worldwide (Dahl 1990) and most amphibians require wetlands for at least part of their life cycle

(Duellman and Trueb 1986). However, there is a lack of conservation genetic studies that focus

primarily on local amphibian populations. It is important to understand how the genetic

variation of local amphibian populations is affected by habitat alteration because this information

will allow understanding of the dynamics of amphibian declines on a local scale.

The present study consists of a population genetic analysis of a local population

of the spotted salamander (*Ambystoma maculatum* (Shaw 1802)) occupying a vernal pool located

on the property of Enterprise South (ES) east of Chattanooga, Tennessee. At present, very few

conservation genetic studies of *A. maculatum* have been published. Previous studies have

focused on connectivity between separate populations (Kinkead et al. 2007; Purrenhage et al.

2009; Zamudio and Wiezcorek 2007). Significant rates of gene flow between populations were

found in these studies. However, populations were located along riparian corridors within 295 meters (the normal dispersal distance for *A. maculatum*; Semlitsch and Bodie 2003) of each other in a linear arrangement. High connectivity was likely explained by a stepping stone effect in these cases. *Ambystoma maculatum* is known to exhibit high breeding site fidelity (Husting 1965; Whitford and Vinegar 1966) and rarely moves more than 295 meters from breeding sites (Semlitsch and Bodie 2003; Semlitsch 1998). These findings suggest that genetic variation in local populations of this species is sensitive to habitat alteration because of low dispersal potential.

Current industrialization of the area near the study wetland includes a recently constructed automobile manufacturing plant and distribution centers for two corporations located to the southeast. Clearing of upland habitat for these facilities began in 2007. Clearing of forested habitat and pavement for parking lots has taken place within dispersal distance (295 meters) of the study wetland. An EDGE effect, where exterior habitat conditions created by habitat alteration penetrate into adjacent undisturbed habitat, is likely to result from an insufficient buffer zone in this case. Large patches of upland salamander habitat have also been decimated by the industrialization. Such activity may have reduced genetic variation among salamanders migrating into the wetland from the south. The upland habitat areas surrounding the wetland were used by the US Army for manufacturing munitions and trinitrotoluene (TNT) storage from the beginning of World War II (1941) until 1977 (ATSDR 2004; Historic American Engineering Record 1984). Storage of TNT likely led to a severe reduction in effective population size (bottleneck) of contamination of upland salamander habitat.

The types of habitat surrounding the study wetland are also likely to have significant effects of the genetic variation of the study population. For purposes of this study, the wetland

can be divided into north, south, west, and east divisions based on cardinal directions. Habitat conditions to the north and east of the wetland are unfavorable for *A. maculatum*. Residential areas that are likely to be unsuitable for use by salamanders lie to the north at a distance of approximately 100 meters from the wetland. Immediately to the east is a ridge separated from the wetland basin by an escarpment. This ridge and escarpment contain poorly drained soils that are not conducive to use by *A. maculatum* for hibernation (Homan et al. 2008). Two wetlands that may serve as sources of migrants are located within dispersal distance to the south and west (Fig. 1). Habitat conditions between these wetlands and the study wetland are mostly favorable for movement of *A. maculatum*. However, clearing for industrialization is located on the eastern portion of the southern boundary of the wetland and may have reduced genetic variation in the southern division. These differential habitat conditions are likely to result in genetic substructure (separate clusters of salamanders characterized by different allele frequencies) with lower genetic variation in divisions with unfavorable habitat conditions in the associated upland regions. *Ambystoma maculatum* reproduces by means of internal fertilization and mating takes place along wetland edges without admixture among salamanders from different directions. Individual salamanders also generally follow the same routes during each breeding migration (Petranka 1998). These characteristics allow for analysis of the effects of differential habitat conditions on a local scale using hypervariable molecular markers (e.g., microsatellites).

Sex-biased dispersal was also investigated in this study because sex ratios are biased in favor of males at the study wetland, which may suggest competition for females. If dispersal is biased in favor of one sex, it indicates that one sex would be responsible for reintroducing genetic variability through gene flow if genetic variation has been lost.

Statement of the Problem

In order to understand the processes that are shaping the genetic profile of the study population, a population genetic analysis is necessary. Past and present habitat disturbance may have depleted genetic diversity or may do so in the future. If this is the case, then the risk of extirpation is high. By uncovering the impact of disturbance on the study population, it will be possible to mitigate loss of genetic diversity and apply the results of this study to future conservation efforts. The primary hypotheses for this study were that genetic variability has been depleted by past and present human use of the area, that the population has undergone a bottleneck resulting from past use, and the effective population size is low (less than 2000 individuals). Additional hypotheses are that genetic variability is higher in the western than in other regions of the wetland, and that the population exhibits male-biased dispersal.

Objectives of the Study

The primary objectives of this study were to assess the genetic variation, recent demographic history, and effective population size of the study population. Additional objectives include comparison of genetic variability between different sections of the wetland based on cardinal directions and examination of potential sex-biased dispersal. The specific objectives of this study were to answer the following questions: i. Has genetic variation (heterozygosity) been depleted from the study population at the majority of loci? ii. Has the population undergone a bottleneck? iii. Is the effective population size low? iv. Is genetic variation greater in the western division of the wetland than in other divisions? v. Does the study population exhibit male-biased dispersal?

Significance of the Study

This study will serve as a guide and baseline for researchers who wish to conduct additional population genetic or ecological studies of the study population or nearby populations. It serves as part of a larger population biology project on the study population. Current and future land owners, developers, and other stakeholders in the Enterprise South region will be able to use this study as a reference to make informed decisions about how to minimize ecological and population genetic impacts of their activities in the area.

CHAPTER 2

METHODOLOGY

Study Organism

The spotted salamander, *Ambystoma maculatum* (Shaw 1802; Figure 1), is a common salamander species of the family Ambystomatidae (mole salamanders) native to eastern North America (Fig. 2; Conant and Collins 1991; Petranka 1998). This species has a requirement for fish-free vernal pools for reproduction and upland deciduous habitat for feeding and hibernation (Petranka 1998; Thompson and Gates 1982). This species utilizes an explosive breeding strategy, with adults migrating to breeding pools in late winter or early spring and remaining for a period of several weeks (Baldauf 1952; Blanchard 1930; Homan et al. 2008; Husting 1965; Petranka 1998; Wilson and Simpson unpublished; Wright and Allen 1909). Local populations can be defined as aggregates of spotted salamanders that share the same breeding pond over time.

Figure 1   Photograph of an adult male specimen of *Ambystoma maculatum*. Collection Number #1535. 21 Dec 2008. Photo courtesy of Joe Simpson.

 A. maculatum is currently listed as "Least Concern" (IUCN Red List 2010). Despite this

fact, valuable information about amphibian conservation can be gained by studying species that

are not imperiled because populations of many such species may be subject to environmental

factors that render them vulnerable to decline and because information about population declines

can be gained by understanding the factors that cause abundant species to decline. Such studies

could provide valuable information about genetic processes contributing to or resulting from

decline of a species and could significantly improve our understanding of how to manage genetic

diversity to prevent it from being lost. Multispecies conservation approaches may also benefit

from studies of non-threatened species due to similar environmental conditions shared by

organisms in the same habitat and similar aspects of biology with respect to imperiled species

(Goldberg and Waits 2010; Whiteley et al. 2006).
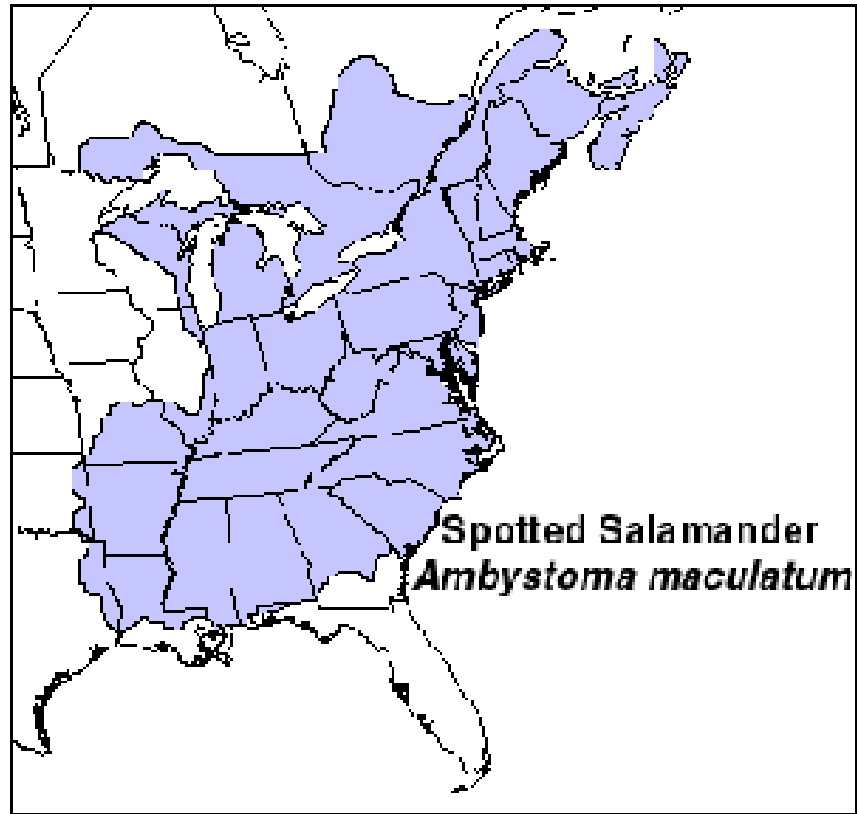
Figure 2 Range map of *Ambystoma maculatum.*

Study Site

In this study, a single ephemeral wetland was sampled for *Ambystoma maculatum*. The study area has an area of 36,421.7 square meters as defined by the average size of the area covered by water during fall and winter. The wetland remains dry from late spring (May) through mid-autumn (October; personal observation). The dominant vegetation of the wetland and upland consists of *Liriodendron tulipifera* (tulip poplar), *Platanus occidentalis* (sycamore), *Fagus grandifolia* (American beech), *Ulmus alata* (winged elm), and several *Quercus* (oak) species. The northeast portion of the wetland is characterized by *Pinus virginiana* (Virginia pine) in the northeast section. The wetland was divided up into North, South, East, and West divisions (Fig. 3) in order to answer the question of whether genetic variation was being

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altered by the residential development to the north and the ridge with unfavorable soils to the
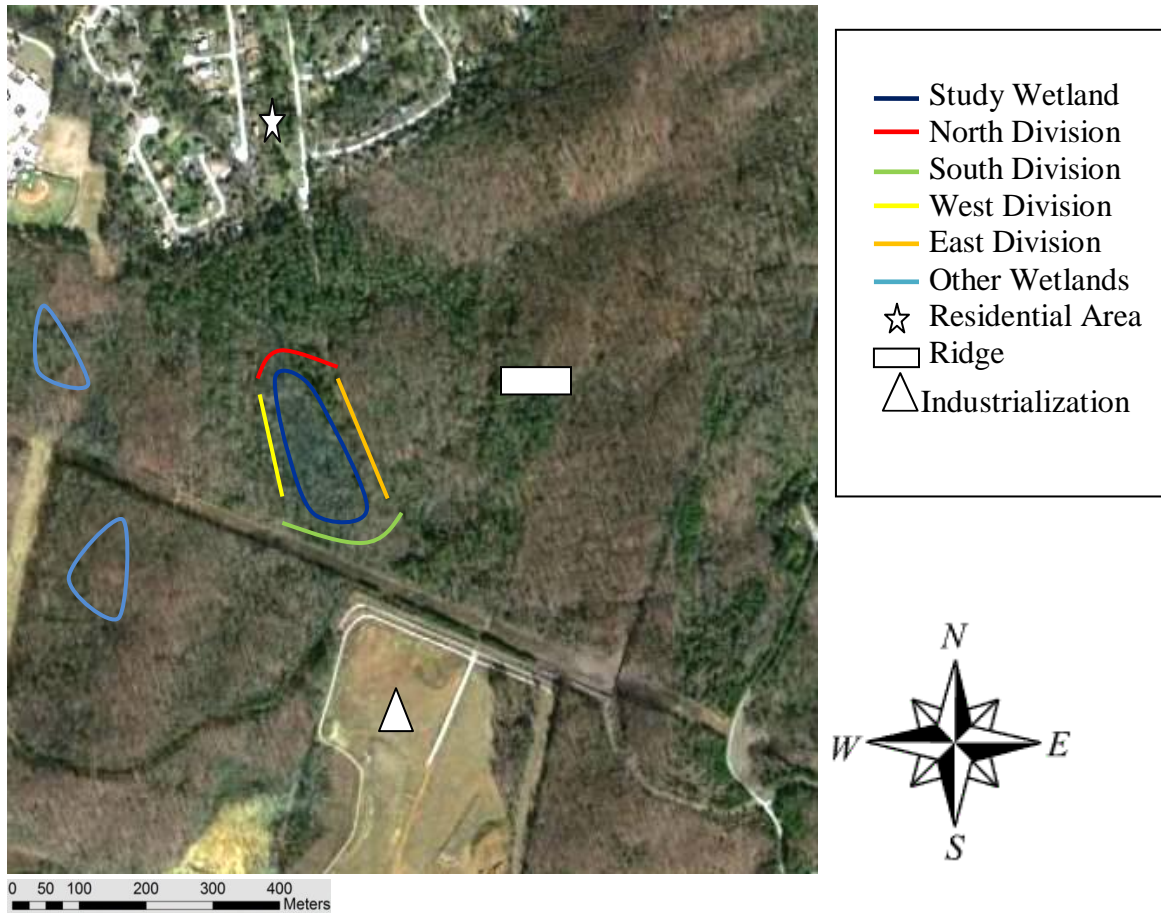
east.

Figure 3   Map showing the study wetland, four divisions, additional wetlands that are sources of migrant salamanders, ridge, residential area to the north, and clearing for industrialization to the southeast. The residential area to the north was expected to reduce genetic variability in the north division because it was located within a 295 m radius (the dispersal distance of *Ambystoma maculatum*) of the study wetland. The ridge to the east was expected to reduce genetic variation in the east division because of its poorly drained soils that were not conducive to hibernation for *A. maculatum*. Two additional wetlands and habitats conducive to salamander hibernation are located to the southwest and west. Although favorable habitat was located to the southwest, clearing for industrialization was expected to reduce genetic variation in the south division because of habitat loss.

Sampling

Drift fences 45 centimeters in height were installed around the wetland with pitfall traps spaced approximately every 7 meters. Sampling was conducted daily from January 2009 to May 2010 with salamanders collected from pitfall traps and transported back to the UTC Herpetology Laboratory for processing when necessary. Salamanders were released within 24 hours if possible and always within 48 hours. The snout-to-vent length (SVL), weight, sex, and capture location of each individual were recorded and placed in the UTC Herpetology Laboratory Database. For each salamander, 1-2 mm of tissue were taken from the distal end of toes according to collection number using scissors sterilized with 100% isopropyl alcohol. Toe clips were stored in 95-100% ethanol at 4-5°C. Tissue samples were retained in the University of Tennessee at Chattanooga Herpetology Laboratory under sequential accession numbers 1-2542. For DNA analysis, 61 samples were randomly chosen with five to eight per sex in each division of the wetland. The sampling design used here was similar to previous amphibian genetic studies. Such studies surveyed multiple populations and typically reported ten to thirty samples per sampling site (e.g., Arens et al. 2007; Burns et al. 2004; Schön et al. 2011). Because one of the goals of this study was to examine the effects of different habitat conditions in the four divisions, the sampling design here consisted of ten to sixteen samples per division. This followed the sampling design of previous amphibian studies in which similar sampled sizes were obtained per site.

DNA Extraction, Quantification, and Concentration

Genomic DNA was extracted from toe clips using the DNeasy Blood and Tissue Kit (Qiagen). In order to facilitate lysis, toe clips were cut into small pieces using sterile scissors.

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The manufacturer's instructions for tissue digestion and elution were followed, except that toe samples were incubated overnight at 65°C in order to ensure complete lysis (A. George, personal communication 01/2010). A second volume of Proteinase K, tissue lysis buffer, and lysis buffer was added to samples that failed to completely digest and such samples were re-incubated overnight. Presence and integrity of DNA in all samples was verified by running them on 1% agarose gels. DNA content of samples was determined using a NanoDrop® spectrophotometer (ThermoScientific).

The DNA concentration of samples was adjusted to 100+/-20 ng/μL via ethanol precipitation as needed (Sambrook and Russell 2001). A 10% volume (of the DNA solution) of 3 M sodium acetate was added to the DNA samples followed by the addition of 2.5 volumes of 100% ethyl alcohol. Samples were frozen at -20°C for 20 min to overnight and centrifuged at 16000 rpm for 15 min to precipitate the DNA. Pellets were washed with 200 μL of 70% ethanol and centrifuged a second time at 16000 rpm for 10 min. The supernatant was discarded and the DNA pellets were dried for 2-4 min in a Speed Vac® (Thermo Scientific) prior to resuspension in 50 μL elution buffer.

Study Loci, PCR, and Genotyping

Population genetic assessment was conducted using thirteen microsatellite loci—
Ama D184, AmaD328, AmaC40, AmaD315, AmaD42, AmaD321, AmaD49, AmaC151, AmaD99, AmaD287, AmaD23, AmaD448, and *AmaD162* (Table 1). Microsatellites were chosen because they have high resolution at small spatial and temporal scales owing to their high mutation rate (Jehle and Arntzen 2002). All loci used in this study were developed at the USGS Leetown

Science Center in Kearneysville, WV. Forward primers were fluorescently labeled with FAM, HEX, or PET.

PCR reactions followed those of Julian et al. (2003) Reagent concentrations included 100 ng of genomic DNA, 1 X PCR Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl; Promega), 2 mM $MgCl_2$ (Promega), 0.25 mM dNTPs (Promega), 0.5 µM each primer, 1.0 U *Taq* Polymerase (Promega), and added dH_2O to bring the final volume up to 10µL. PCR cycles were as follows: an initial denaturation step at 94^oC for 5 min followed by 35 cycles of denaturation at 94^oC for 45 s, annealing at 58^oC for 45 s, elongation at 72^oC for 1 min 30 s, and a final extension step at 72^oC for 5 min. PCR reactions and genotyping were performed at the Leetown Science Center in Kearneysville, WV, USA.

Table 1 Repeat motifs, size ranges of PCR products and original references for the thirteen tetranucleotide microsatellite loci used in this study. Repeat motifs and size ranges for the first ten loci were obtained from Julian et al. (2003). These loci were deposited in GenBank under accession numbers AF520747-AF520759 (www.ncbi.nlm.nih.gov/genbank/). *AmaD23, AmaD162,* and *AmaD448* have been developed at the Leetown Science Center in Kearneysville, WV and are currently in press.

Locus	Repeat Motif	Size Range (bp)	Reference
AmaC40	(TACA)7	155-175	Julian et al. (2003)
AmaC151	(TACA)3…(TACA)4	220-425	Julian et al. (2003)
AmaD42	(TAGA)4…(TAGA)3	125-160	Julian et al. (2003)
AmaD49	(TAGA)11	100-180	Julian et al. (2003)
AmaD99	(TAGA)12	125-210	Julian et al. (2003)
AmaD184	(TATC)11	115-175	Julian et al. (2003)
AmaD287	(TATC)16	170-215	Julian et al. (2003)
AmaD315	(TATC)18	195-255	Julian et al. (2003)
AmaD321	(TATC)14	120-175	Julian et al. (2003)
AmaD328	(TAGA)15	260-305	Julian et al. (2003)
AmaD23	Not available	Not available	King (unpublished)
AmaD162	Not available	Not available	King (unpublished)
AmaD448	Not available	Not available	King (unpublished)

Alleles were scored by capillary electrophoresis using an ABI Prism 3100 Genetic

Analyzer (Applied Biosystems) with the TAMRA-500 size standard and the software

GeneMapper 3.0 (ABI). Prior to electrophoresis, PCR products were denatured with 12µL

formamide and heated to 95°C for 5 min. Peaks with heights less than the default stutter ratio of

0.20 were considered stutter and therefore not scored. Ambiguous genotypes were resolved by

comparison with previously reported size ranges for each allele.

Statistical Analysis

Heterozygosity and Allelic Richness

For each locus, expected heterozygosity was calculated using the Hardy-Weinberg

equation. Observed heterozygosities at each locus were determined by calculating the

percentage of individuals with heterozygous genotypes at that locus. These calculations were

performed using the software GENALEX 6.0 (Peakall and Smouse 2006). Allelic richness was

calculated using the statistical technique of rarefaction in order to correct for population size.

Rarefaction is a technique used to estimate allelic richness using rarefaction curves which show

how expected allelic richness becomes larger with increased sample size (analogous to species-

area curves in which the expected number of species becomes larger with increased area). These

calculations were performed using the program HP-RARE (Kalinowski 2005). Although allelic

richness and expected heterozygosity are similar measures of genetic diversity, both were used

for comparative purposes because allelic richness is strongly affected by sample size while

expected heterozygosity is less affected by sample size (Kalinowski 2004).

Hardy-Weinberg and Linkage Equilibrium

Tests for Hardy-Weinberg equilibrium were used to answer the question of whether

genetic variation, as measured by heterozygosity, was lost at any locus. Heterozygote

deficiencies generate deviations from HW expectations. These tests were performed using Markov chain Monte Carlo (MCMC) modifications of Fisher's exact test for multiple alleles (Guo and Thompson 1992; Ho: union of gametes was random) in GENEPOP 4.0 (Raymond and Rousset 1995). Because frequencies for most alleles were small, exact tests were more reliable than standard goodness-of-fit chi-square tests in this case. Significance was assessed with 1000 batches of 10000 iterations following 10000 dememorization (burn-in) steps. Exact P-values for each locus were computed by dividing the number of iterations with expected genotype count probabilities lower than that observed for the sample by the total number of iterations. F_{IS} values were computed for each locus. F_{IS} is a within-population inbreeding coefficient that measures loss of heterozygosity. Theoretical values of F_{IS} range from -1 to 1, with higher values indicating greater loss of heterozygosity. Deviations from HWE resulting from heterozygote deficiencies indicate F_{IS} values that are greater than expected under HWE. Sequential Bonferroni corrections (Rice 1989) were applied to this and all other multiple comparison tests in order to maintain a table-wide significance level of 0.05. Sequential Bonferroni corrections are less conservative than traditional Bonferroni corrections and are less likely to lead to false negatives (Holm 1979).

Testing and Correction for Null Alleles

Null alleles are a problem that may arise when microsatellite markers are used for population genetic studies. These are alleles that fail to amplify during PCR usually because of mutations in the primer binding sequences. In addition, large microsatellite alleles may not amplify as efficiently as smaller ones (allelic dropout) and slippage of DNA polymerase can result in scoring errors due to stutter. Null alleles may lead to underestimates of heterozygosity and can affect the results of tests that rely on allele frequency input data. A permutation test was

used to determine if such problems with the markers were present. Average values of homozygosity and 95% confidence intervals were calculated for 1000 randomized data sets for all loci based on the observed heterozygosity for each allele at each locus. Observed homozygosity values greater than the upper limit of their 95% confidence intervals (homozygote excess) were considered to be evidence for scoring errors due to null alleles at that locus. Large allele dropout is suggested by excess homozygosity that is biased toward the ends of the allele size distribution and scoring errors due to stuttering were suggested by homozygote excesses with respect to alleles that differ in size by a single repeat unit. These analyses were conducted using the program MICRO-CHECKER (Oosterhaut et al. 2004).

Evidence for null alleles was found at three loci (see Results) and a method of statistical correction developed by Brookfield (1996) was used to estimate the frequencies of the null alleles and to adjust the frequencies of amplified alleles accordingly. In order to determine whether including probable null alleles in the data set would eliminate deviations from Hardy-Weinberg equilibrium, the data set was re-analyzed twice for deviations from HWE. Probable null alleles were recoded as missing data in one analysis and assigned an identity consistent with the repeat motif of the locus in the other. Neither method eliminated deviations from HWE at any locus so the original uncorrected data set was used for all subsequent analyses. One locus (*AmaD99*) was excluded from further analysis because it exhibited evidence of stutter.

All pairs of loci were tested for linkage disequilibrium (Ho: genotypes at all loci are independent of genotypes at other loci) using the log-likelihood ratio test (Weir 1996) implemented in GENEPOP 4.0 with the MCMC exact test modification (Raymond and Rousset 1995). These tests were done for two reasons. First, linkage disequilibrium reduces the number of independent loci available for analysis. Second, linkage disequilibrium can be the result of

population structure caused by migrant individuals from nearby populations and may indicate differential genetic variation between different divisions of the wetland (Beebee and Rowe 2004). For all pairs of loci, contingency tables of the numbers of individuals with each genotype were generated. Tables were randomly permuted in a series of 1000 batches each with 10000 randomizations preceded by 10000 burn-in steps.

Population Bottleneck

The program BOTTLENECK 1.2.02 (Piry et al. 1999) was used to answer the question of whether the population had undergone a bottleneck resulting from ammunition and TNT storage. The heterozygosity excess method implemented in BOTTLENECK was used to test for an excess of expected heterozygosity under Hardy-Weinberg relative to expected heterozygosity under mutation-drift equilibrium. The data were analyzed according to the stepwise mutation model (SMM) and the two-phase model (TPM) of microsatellite evolution. These models were selected because microsatellite loci generally follow the SMM, in which new alleles are generated by the addition or deletion of repeat units (Jarne and Lagoda 1996; Valdes et al. 1993). The TPM (Di Rienzo et al. 1994) is a hybrid mutation model of the SMM and infinite allele model (IAM). Most other types of loci follow the IAM, in which mutations generate novel alleles (Kimura and Crow 1964). The one-tailed Wilcoxon sign-rank test ($\alpha = 0.05$) was used to test for an excess of heterozygosity under the null hypothesis of no heterozygosity excess across loci.

Effective Population Size

There are many ways to estimate N_e from genetic data, but the choice of methods for this study was limited because only a single genetic sample was available. The most powerful approaches involve temporal samples taken at least one generation apart (Beebee and Rowe 2004). Only a single genetic sample was available for the present study and thus a method that relies on such had to be utilized. The method used here was the linkage disequilibrium method which calculates N_e based on the rate of decay in linkage equilibrium (Hill 1981). 95% confidence intervals were calculated from a null distribution generated by bootstrapping. This method was selected as the method of choice because it is the only one that provided reasonable (non-infinite) confidence intervals.

Population Structure

In order to test for population structure resulting from differential genetic variation between the four divisions of the wetland, two tests were used. Tests for population structure were used because genetic differentiation between any two divisions will generate separate genetic clusters. The first test used for population structure was a Bayesian clustering algorithm implemented in STRUCTURE 2.3.1 (Hubisz et al. 2007) was used to determine the most probable number of genetic clusters in the study population (K). This algorithm estimates Pr (K/X), which is the mean of the posterior probability distribution representing the probability of K given the genotypes in the sample. The admixture model implemented in STRUCTURE allows for each individual to derive portions of his/her genome from any cluster (Falush et al. 2003). The MCMC scheme was run five times for each value of K from 2 to 7 with 10000 steps

following a burn-in length of 10000 for each run. The most probable number of genotypic

clusters (K) was estimated using the ΔK correction of Evanno et al. (2005).

An exact test for genic differentiation implemented in GENEPOP 4.0 was used to test for

differentiation of allele frequencies between the four divisions of the wetland. Randomized

contingency tables of allele frequency distributions were generated using a MCMC scheme

(10000 dememorizations (burn-in steps) and 1000 batches of 10000 iterations).

Sex-Biased Dispersal

Testing for sex-biased dispersal was done using the randomization test for sex-biased

dispersal implemented in FSTAT 2.9.3 (Goudet 2001). Estimates of pairwise relatedness, H_o,

H_e, and F_{IS} were randomized 10000 times in order to generate a distribution of these values

under the null hypothesis of symmetrical dispersal. The relatedness estimator of Queller and

Goodnight (1989) was used to estimate pairwise relatedness between individuals. Because F

statistics are a less sensitive indicator of sex-biased dispersal than other methods that rely on

inter-population comparisons (Goudet et al. 2002), STRUCTURE analyses were repeated

separately for males and females in order to detect migrant and resident genetic clusters that may

result from one sex dispersing more often than the other sex. Values of K from 2 to 8 were run

five times.

CHAPTER 3

RESULTS

Findings

Heterozygosity and Allelic Richness

All loci were polymorphic based on the 95% criterion, with the number of alleles per locus ranging from five to thirteen. Observed heterozygosities per locus ranged from 0.176 to 0.865 and expected hetrozygosities ranged from 0.432 to 0.842. Allelic richness values per locus ranged from 2.62 to 6.81 (Table 2). The total number of alleles across all loci and divisions was 107. The number of private alleles ranged from four in the South division to eight in the East division.

Hardy-Weinberg and Linkage Equilbrium

Departures from HWE were observed at four loci, with all remaining significant after Bonferroni correction (Table 3). Three of these (*AmaD23*, *AmaD99*, and *AmaD287*) were found to have evidence of null alleles. Correction for null alleles did not appreciably change genetic diversity indices and did not improve departures from HWE. Hence, all results presented here are for the uncorrected data set. No evidence of null alleles was found at *AmaD49* despite a departure from HWE. *AmaD99* also exhibited evidence of scoring errors due to stutter based on the results of MICROCHECKER analysis. There was no significant linkage disequilibrium between any pair of loci.

Population History and Effective Size

Following Bonferroni correction, there were no loci that exhibited evidence of expected heterozygosity excess relative to heterozygosity expected at mutation-drift equilibrium. The Wilcoxon sign-rank test indicated no significant evidence of overall heterozygosity excess in the local population (P-value = 0.48). Furthermore, there was no significant loss of rare alleles as indicates by the absence of a mode shift of the allele frequency distribution toward more common alleles (Figure 4). Based on the linkage disequilibrium method, N_e was 181 individuals (95% CI 119-352).

Table 2   Number of alleles (N_a), H_o (with standard errors), H_e (with standard errors), and allelic richness (AR) for each locus. Values are pooled across all wetland divisions. Means for all loci are shown in the bottom row.

Locus	*Na*	*Ho* ($^+/.$SE)	*He* ($^+/.$SE)	AR
AmaC40	6	0.464 (0.084)	0.432 (0.044)	2.62
AmaC151	10	0.703 (0.030)	0.741 (0.020)	4.8
AmaD23	7	0.506 (0.086)	0.687 (0.044)	4.53
AmaD42	5	0.758 (0.085)	0.661 (0.008)	3.63
AmaD49	8	0.781 (0.074)	0.809 (0.011)	5.81
AmaD99	5	0.176 (0.107)	0.529 (0.112)	3.15
AmaD162	8	0.582 (0.201)	0.561 (0.188)	3.84
AmaD184	13	0.850 (0.063)	0.842 (0.015)	5.04
AmaD287	8	0.510 (0.048)	0.642 (0.052)	6.81
AmaD315	9	0.479 (0.074)	0.505 (0.069)	3.45
AmaD321	9	0.563 (0.057)	0.790 (0.016)	5.7
AmaD328	12	0.732 (0.052)	0.728 (0.018)	5.33
AmaD448	7	0.865 (0.059)	0.777 (0.018)	5.21
Mean	8.417	0.613 (0.078)	0.717 (0.034)	4.61

Table 3 F_{IS} values (inbreeding coefficients) and P-values for Hardy-Weinberg exact tests. F_{IS} is a measure of the discrepancy between the observed and expected heterozygosity. Boldface indicates significant P-values following sequential Bonferroni correction to maintain a table-wide alpha level of 0.05. Deviations from Hardy-Weinberg equilibrium indicate F_{IS} values greater than expected under conformance to Hardy-Weinberg expectations caused by heterozygote deficits.

Locus	F_{IS} (+/.SE)	Hardy-Weinberg P-value
AmaC40	-0.0092 (+/.0.0032)	0.633
AmaC151	0.0947 (+/.0.0026)	0.1658
AmaD23	0.0315 (+/.0.0000)	**>0.001***
AmaD42	-0.1684 (+/.0.0003)	0.9784
AmaD49	0.0928 (+/.0.0001)	**0.0028**
AmaD99	-0.0541 (+/.0.0005)	**0.0488*#**
AmaD162	0.0029 (+/.0.0017)	0.4764
AmaD184	0.0252 (+/.0.0027)	0.2185
AmaD287	0.0549 (+/.0.0005)	**0.0175***
AmaD315	0.0453 (+/.0.0032)	0.2291
AmaD321	0.0061 (+/.0.0023)	0.6084
AmaD328	0.0159 (+/.0.0039)	0.5323
AmaD448	-0.0268 (+/.0.0016)	0.4542

* = evidence of null alleles
# = evidence of scoring errors due to stutter
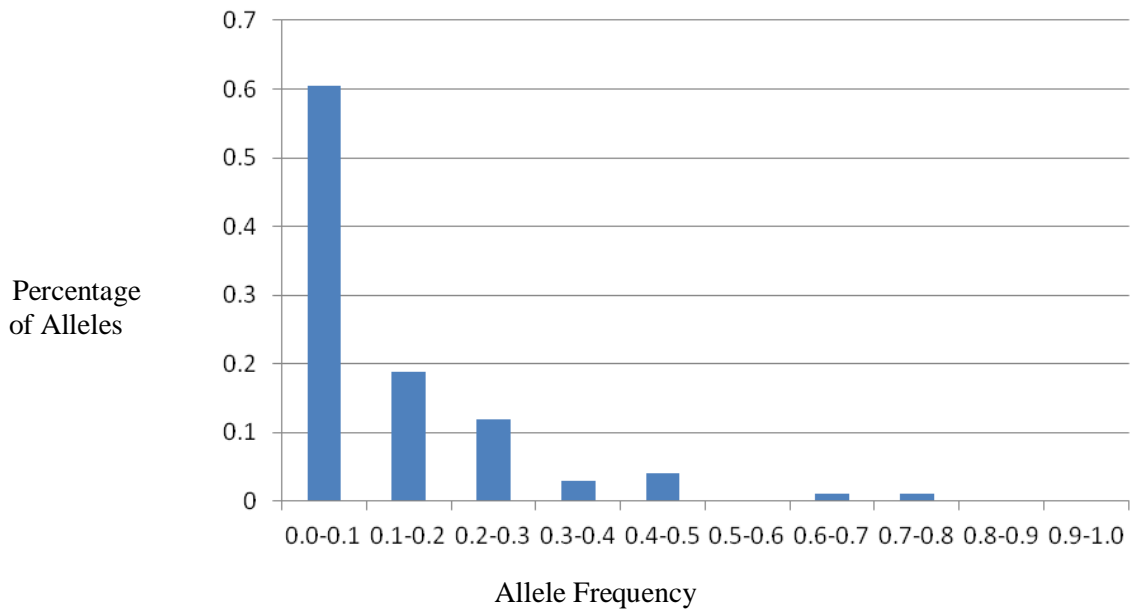
Figure 4   Bar graph showing percentages of alleles in each allele frequency class. Most alleles (61 percent) had a frequency less than 0.1. This is indicative of the absence of a population bottleneck caused by TNT and ammunition storage in the upland area around the wetland. Bottlenecks typically result in loss of low frequency alleles.

Population Structure

The Bayesian clustering algorithm implemented in STRUCTURE did not detect any genetic clusters within the local population (Figure 5). Posterior probabilities remained consistent across different runs at the same value of K and across different values of K, which indicates panmixia. All individuals derived equal portions of their genome from the four wetland clusters (Figure 5). The exact test of genetic differentiation between the four divisions of the wetland did not detect any significant differentiation between any pairs of divisions.

Figure 5 STRUCTURE bar graph showing panmixia for the study population. Each vertical bar represents a single individual out of 61. Each horizontal shaded bar represents the North, West, South, and East division, respectively, from top to bottom. Each individual is equally assigned (membership coefficients ~0.25) to all four divisions. This indicates that there is no detectable genetic clusters nor is there any reduction in genetic variation in the north and east divisions.

Sex-Biased Dispersal

Genetic diversity indices (pairwise relatedness, H_o, H_e, and F_{IS}) were not significantly different between males and females (Table 4). STRUCTURE analyses did not detect separate migrant clusters for males or females. Both of these results are indicative of the absence of male-biased dispersal.

Table 4   A comparison of relatedness, F_{IS}, observedheterozygosity, and expected heterozygosity between males and females. Numbers shown for these statistics are averages across all loci and all wetland divisions. The absence of significant male-biased dispersal is indicated by the absence of significant difference between males and females for these statistics.

Statistic	Males	Females	P-value
F_{IS}	0.099	0.101	0.49
Relatedness	0.011	0.020	0.38
H_o	0.628	0.624	0.29
H_e	0.719	0.694	0.15

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Summary and Implication of the Findings

Heterozygosity and Allelic Richness

The majority of loci (*AmaC40, AmaC151, AmaD42, AmaD162, AmaD184, AmaD315,*

AmaD321, AmaD328, and *AmaD448*) exhibited high heterozygosity as indicated by conformance to

Hardy-Weinberg expectations (Table 3). Such levels of heterozygosity were inconsistent with the

hypothesis of reductions in genetic variability. Habitat alterations such as previous use of upland

habitat around the wetland for ammunition and TNT storage (1941-1977) and recent construction

of an automobile manufacturing plant and distribution centers for two corporations were

expected to cause significant reductions at heterozygosity for most or all loci. However, the

amount of time that has passed since construction of the automobile manufacturing plant and

distribution centers is likely to be insufficient to result in detectable reductions in genetic

variation. The absence of genetic differentiation between the divisions of the wetland (Fig. 5)

indicates that loss of heterozygosity is distributed equally throughout the wetland. If current

industrialization was reducing genetic variation, then lower heterozygosity would have been

expected in the southern division of the wetland (Fig. 1). This would have been expected to

result in genetic differentiation of the southern division from the western division which had

more favorable habitat conditions. Three loci (*AmaD23, AmaD49,* and *AmaD287*) were not in

accordance with Hardy-Weinberg expectations due to loss of heterozygosity (Table 3). Results

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for these loci were consistent with the hypothesis of reduced genetic variation. The deviations

from HWE at *AmaD23* and *AmaD287* were not explained by null alleles. Therefore these

departures from HWE were caused by observed heterozygosities being significantly lower than

expected heterozygosities because of biological reasons.

The neutrality of microsatellites implies that genetic drift, and not natural selection, has

been the main evolutionary force causing loss of heterozygosity at *AmaD23, AmaD49,* and

AmaD287. Possible explanations for the departures from HWE at these three loci include

lingering loss of heterozygosity from a past population decline caused by manufacturing and

storage of munitions and TNT, loss of heterozygosity from natural fluctuations in population size

or dispersal, and early effects of an impending loss of heterozygosity resulting from previous or

present habitat alteration.

There was no excess of heterozygosity expected under Hardy-Weinberg relative to

heterozygosity expected under mutation-drift equilibrium. Most alleles had a frequency less than

0.1 (Fig. 4). Both of these findings indicate demographic stability over time. This was

unexpected and did not support the hypothesis of a population bottleneck. Storage of munitions

and TNT was hypothesized to have caused a severe reduction in population size as a result of

contamination of upland habitat and dispersal routes.

Amphibian studies in which bottlenecks have been described or detected have focused on

the effects of landscape alteration (Allentoft et al. 2009; Richter et al. 2009) or founder effects

(Ficetola et al. 2008; Peacock et al. 2009) rather than chemical pollution or material storage. It is

possible that chemical pollution may have lesser impacts on the genetic variability of amphibian

populations than other forms of disturbance (DiBattista 2008). This may explain the high

heterozygosity at most loci and demographic stability over time seen in this study. In addition,

patches of favorable habitat may have been interspersed with areas of TNT and ammunition storage. The amount of time that has passed since current industrialization of the area began is probably not sufficient to result a magnitude of genetic drift that can be detected with bottleneck tests. Amphibians have been suggested to use patches of favorable habitat as refugia when conditions are unfavorable (Dodd and Smith 2003). Despite results of the bottleneck tests, it is likely that past usage of the upland habitat was the cause of heterozygosity losses at *AmaD23, AmaD49,* and AmaD287. Such losses were not of sufficient magnitude to be detected by bottleneck tests (Allentoft et al. 2009; Richter et al. 2009).

Another possible explanation for the aforementioned loss of genetic variation is that heterozygosity may fluctuate as a result of natural population cycles, with it declining after periods of lower population sizes and lower dispersal rates. Available evidence suggests that natural oscillations in population size are common occurrences in pond-breeding amphibians (Richter et al. 2003; Richter and Seigel 2002; Semlitsch et al. 1996; Wells 2007; Whiteman and Wissinger 2005). Differential reproductive and mortality rates between years and abiotic factors such as yearly fluctuations in temperature and rainfall are likely to play roles (Halliday 2005; Pechmann 2003). Rates of intermigration between the study wetland and nearby wetlands appear to fluctuate between years based on recapture rates (Thomas Wilson, Univ. of Tenn. at Chatt., personal communication 22 Feb 2012). Such oscillations in dispersal may also have had an effect on genetic diversity due to lower levels of gene flow during periods of less dispersal. Distinguishing between natural population oscillations and true population declines is a challenge because they can have very similar effects on genetic variability and population size (Pechmann et al. 1991; Pechmann and Wilbur 1994). However, this distinction is important because true population declines necessitate conservation efforts in order to preserve genetic

diversity and prevent further declines in population size. Long-term monitoring of census

population size, effective population size, and genetic diversity is necessary in order to make

such a distinction and to determine the extent to which population cycles affect the genetic

variability of the study population.

It is possible that genetic diversity may be in the process of decline at the present time for

both the past habitat disturbance and natural population cycle scenarios. The elapsed time

between the period of heaviest disturbance or most recent natural decline and the present time

may not be sufficient for manifestations of the greatest effects on genetic variation. Latency

times between disturbances and their genetic effects are not well studied in amphibians and most

studies to date have focused on the temporal sensitivity of inter-population genetic distance

measures (Keyghobadi et al. 2005; Landguth et al. 2010; Murphy et al. 2010). The degree of

disturbance may affect time to fixation of alleles, with greater degrees of disturbances shortening

such time (Ezard and Travis 2006). The exact factors that affect the rate at which disturbances

become detectable via genetic methods may differ substantially from one population to another.

Variation in N_e, genetic variability, dispersal ability, and generation time may play significant

roles (Murphy et al. 2008).

Although most loci did not exhibit a significant reduction in heterozygosity, loss of

heterozygosity at three loci may have been caused by past usage of the area around the study

wetland by the US Army. Further loss of heterozygosity at these loci and loss of heterozygosity

at the other eight loci is likely to result from recent construction of an automobile manufacturing

plant and distribution centers for two corporations because of upland habitat loss and EDGE

effects. This means that adequate buffer zones of at least 295 meters should be constructed

between the study wetland in order to mitigate existing loss of and help prevent future loss of genetic variation.

Effective Population Size

The effective population size of 181 (95% CI 119-352) was low but expected. This result supported the hypothesis of a small effective size. N_e values for studied amphibian populations range from 30 to 2000 but tend to be in the lower half of this range. Although this value was similar to or higher than the N_e values reported in the literature for most studied amphibian populations (Beebee 2009; Brede and Beebee 2006; Easteal 1985; Funk et al. 1999; Jehle et al. 2001; Schmeller and Merilä 2007), it is considered low because the ratio of N_e to the census population size (N) is less than desirable for population persistence. The average estimate of N for the study population was 1,877 with 95% confidence intervals of 1,248-3,785 (Wilson and Simpson, unpublished). Both the estimated N_e and associated confidence intervals (119-352) have N_e/N ratios of approximately 0.1. Demographic predictor models of N_e typically predict a N_e/N ratio of 0.25 to 0.75 and such ratios are more desirable for conservation purposes than lower ratios (Nunney 1993). Previous studies of N_e in amphibians have generally been conducted on populations with much smaller census sizes. This likely explains the lower N_e values found in many other studied populations as N_e/N ratios have been similar to or higher than the one discussed here.

Sample sizes per population have not been reported in most studies which it difficult to evaluate the effects of sample size on N_e for this and other studies. Schmeller and Merilä (2007) report sample sizes of 58 and 80 and N_e values of 97 and 13, respectively, using the same

methodology for two populations of *Rana temporaria* (common frog). However, N_e/N ratios were higher (1.67 and 0.23, respectively).

The N_e value of 181 indicates that the genetic response to current industrialization of the area will be as if the population was composed of 181 individuals rather than the census number of individuals. The apparent discrepancy between the high levels of heterozygosity at most loci and N_e may be explained by a consistently low value of N_e relative to the census size or oscillations in census population size. There is typically a positive relationship between levels of heterozgyosity and effective population size (Frankham 1996), but N_e is extremely sensitive to variations in census size and may not recover following declines in census size (Frankham 1995b; Kalinowski and Waples 2002; Vucetich et al. 1997; Waples 2002). Thus, a low effective size dating from the time before ammunition and TNT storage or fluctuating population sizes over time may explain the low N_e despite relatively high levels of heterozygosity at most loci. It is also possible that N_e may be in the process of declining along with heterozygosity following ammunition and TNT storage.

An important consideration is that N_e does not represent the minimum number of individuals required to maintain the population. A value of 181 does not imply that only 181 individuals are necessary to maintain the study population. Rather, this is the adjusted population size after the number of breeding and non-breeding individuals of both sexes have been considered. Population genetic changes depend on N_e rather than the actual census size because only breeding individuals contribute genetic material to the next generation. MVP, which is the number of individuals required to maintain the population, is a concept that is related to but distinct from N_e. This is a distinction that should be considered by development projects and conservation plans. Continual monitoring of N_e is necessary because a lower value

as found in this study implies that population genetic consequences of habitat disturbance will be more pronounced than if N_e was larger (Ellstrand and Elam 1993). This finding necessitates a careful conservation plan that should include sustainable development of Enterprise South. MVP should be determined for the population in future studies because it will indicate whether N_e is below a threshold for population persistence and will be necessary for conducting population viability analyses (Boyce 1992; Reed et al. 2003; Thomas 1990). Future studies of N_e at the current study site and nearby wetland sites should provide a more comprehensive understanding of how N_e may change over time and help to determine the factors that have been most important in shaping N_e for the population. N_e is important for measuring the genetic health of a population and therefore a more complete understanding of this parameter will be very helpful in further understanding the population genetic structure and dynamics of the study population. Multiple temporal samples using both genetic and demographic data should provide a more robust estimate of N_e as it tends to exhibit temporal variation and, as noted above, is sensitive to fluctuations in census population size (Beaumont 2003; Vucetich and Waite 1999; Wright 1938).

Gene Flow and Population Substructure

STRUCTURE analyses indicated no differentiation of allele frequencies between the North, East, South, or West divisions of the wetland (Fig. 5). The results of the exact tests of population differentiation are consistent with the results of the STRUCTURE analyses. Together these results did not support the hypothesis of greater genetic variability in the West division of the wetland. Unexpectedly, the ridge with unfavorable soils to the east does not appear to be reducing genetic variation. Observed migration rates of salamanders do not differ between the

divisions of the wetland (Wilson and Simpson, unpublished). Together these results suggest that patches of well-drained soils conducive to salamander hibernation are interspersed with patches of unfavorable poorly drained soils.

Recent construction of an automobile manufacturing plant and distribution centers does not appear to be reducing heterozygosity in the South division. If this was the case, lower heterozygosity may have resulted and produced significant genetic differentiation of this division from the others. However, this result should be considered inconclusive because the amount of time that has elapsed since clearing for industrialization began may be insufficient to result in detectable loss of heterozygosity. Continued monitoring of genetic variation partitioned between the four divisions of the wetland is necessary in order to assess the future impact of such activity.

Based on the results of STRUCTURE analyses, it appears that no new genetic variation is being introduced into the study population at a detectable level. The lack of significant linkage disequilibrium implies that allele frequencies in the neighboring local populations are similar to those in the sampled population because genetic differentiation between nearby populations typically results in significant linkage disequilibrium (Hartl and Clark 2007). Although the greatest dispersal distance for *A. maculatum* is around 295 m, gene flow often has a homogenizing effect on allele frequencies and identities when local amphibian populations are separated by distances of less than five kilometers. Very small numbers of migrants are often sufficient to prevent divergence of allele frequencies (Scribner et al. 2001) and small numbers of salamanders migrate farther than 295 meters away from breeding ponds (Semlitsch and Bodie 2003). Nearby populations are thus unlikely to introduce significant numbers of new alleles under the current conditions. In the event of severe loss of genetic variation, however,

reintroduction of genetic variation from nearby populations (genetic rescue) may be possible if it is preserved in those populations (Tallmon et al. 2004).

Sex-Biased Dispersal

The data in the present study do not appear to be indicative of sex-biased dispersal as indicated by a lack of significant differences between observed heterozygosity, expected heterozygosity, inbreeding coefficients (F_{IS}), or pairwise relatedness values between individuals (Table 4). Although F_{IS} suffers from limited power to detect sex-biased dispersal, non-significant sex differences for other measures of genetic diversity increase confidence that there is indeed no detectable bias in dispersal. The lack of observable population structure for either sex is also consistent with symmetric dispersal as the dispersing sex would be composed of immigrant and resident populations if dispersal was asymmetrical. The slightly male-biased sex ratio and earlier arrival of males observed at the study site (Wilson and Simpson, unpublished data) indicates that there may be a small but insignificant male bias in dispersal which may simply be the result of greater populations size for males.

Because of inconsistent results reported for sex-biased dispersal in amphibians (Austin et al. 2003; Cabe et al. 2007; Knopp and Merilä 2009; Lampert et al. 2003; Liegbold et al. 2011; Palo et al. 2004) and because proximate and ultimate causes vary widely even among closely related taxa (Handley and Perrin 2007), it is difficult to interpret the results of the present study in the larger context. There is currently too little information on SBD in the family Ambystomatidae to draw any general conclusions. Further research dealing with this topic is necessary to uncover general patterns of sex-biased dispersal in amphibians and underlying evolutionary and ecological causes.

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Conservation Implications

Although heterozygosity levels are high at most loci and the population appears to have been demographically stable through time, low heterozygosity levels at three loci may have resulted from previous habitat disturbance. Current industrialization has already decimated large patches of upland salamander habitat and led to a potential EDGE effect because of the insufficient buffer zone. Resulting impacts on the genetic variation of the study population may be severe because of the low effective population size. Construction of a new road along the southern edge of the wetland is likely to impede migration of salamanders to and from the wetland and is likely to also result in reductions in population size and genetic variability. A buffer zone of at least 295 meters should be constructed in order to minimize negative effects on the genetic variation of the study population.

Conclusions

Observed levels of heterozygosity at most loci and the lack of a bottleneck signature were inconsistent with the hypotheses of reductions in heterozygosity at the majority of loci resulting from storage of ammunition and TNT near the study wetland. Declines in heterozygosity at three loci, however, may have been caused by such storage. Possible temporal scenarios include a slight decrease in genetic variability from past habitat alteration, future decreases in genetic variability that have yet to result from the same period of habitat alteration, fluctuations in genetic variability resulting from natural oscillations in population size, and maintenance of a consistent level of genetic variability over time. The lack of observable genetic clusters was not consistent with the hypothesis of differential genetic variation based on cardinal directions. This indicates that the population's genetic variation is not significantly reduced by unfavorable

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habitat conditions to the north and east. Current industrialization to the southeast does not appear to have reduced genetic variation at the present time but this may change in the future. These results also indicate that no new genetic variation is being introduced at a detectable level. The lack of male-biased dispersal was inconsistent with the hypothesis of male-biased dispersal and indicates that neither sex is disproportionately contributing to gene flow between the study wetland and the two wetlands within dispersal distance. Minimization of the EDGE effect and maintenance of upland habitat conditions should help to prevent further loss of heterozygosity and possible extirpation.

Future Directions and Recommendations

Further studies are needed in order to gain a more complete understanding of the population genetic profile of the study population and the factors that have been most important in shaping its genetic diversity. Future monitoring of population genetic dynamics is necessary to gain a more complete understanding of how both past and present habitat disturbances have affected the study population and the effects that present disturbances may have in the future. Nearby wetlands should be evaluated using the same techniques in order to uncover how population genetic dynamics operate at the metapopulations level. Further research should ultimately seek to apply any findings to conservation of the study population.

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VITA

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