POPULATION GENETIC STRUCTURE OF THE RED-SPOTTED NEWT (NOTOPHTHALMUS VIRIDESCENS VIRIDESCENS) IN VIRGINIA

by

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A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at George Mason University

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ABSTRACT

POPULATION GENETIC STRUCTURE OF THE RED-SPOTTED NEWT (NOTOPHTHALMUS VIRIDESCENS VIRIDESCENS) IN VIRGINIA

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The red-spotted newt (*Notophthalmus viridescens viridescens*) is ubiquitous throughout eastern North America. Despite its commonness, gaps exist in our understanding of its population structure. The hypothesis, first proposed in the 1970's, that the red-spotted newt exists as an intrametapopulational panmixis dependent on high levels of connectivity for its long- term persistence has not been adequately tested using the genetic tools developed since that time. Instead, genetic analyses to date have focused on the population structures of the rarer subspecies of *N. viridescens* or on the phylogenetic relationships between these subspecies. To my knowledge, this is the first study of population structure conducted specifically on the red-spotted newt.

To evaluate the genetic population structure, I collected sequence data on the mitochondrial D-loop and the flanking $tRNA^{Pro}$ and $tRNA^{Phe}$ genes from nine populations of newts located in the Shenandoah Mountains west of Harrisonburg, Virginia and in the Massanutten Mountains. The lack of sequence differentiation between these populations indicates a collection of breeding populations connected through gene flow, supporting the premise of a metapopulation structure. This connectivity was maintained even

though the ponds were located on separate mountain ridges. Presumably the contiguous, forested habitat between ponds allowed unimpeded dispersal between ponds. The detection of unique haplotypes in an isolated Massanutten Mountain pond may indicate a recent disruption in gene flow and warrants further investigation. The level of habitat disturbance seen in the Shenandoah Valley isolating this pond would be prohibitive to dispersal.

An evaluation of microsatellite markers previously published and those developed for this study revealed the likelihood of polyploidy in these populations. Triploidy in *N. viridescens* had been observed previously through cytogenetic analysis; however, the tetraploidy observed in this study had not previously been documented. It is unknown whether this is unique to the populations in this study.

The long-term survival of the red-spotted newt is dependent on the connectivity of the sub-populations of the regional metapopulation. As such, conservation efforts should focus on maintaining connectivity between populations. This study has called attention to the need for further genetic research to identify populations at risk of genetic isolation.

CHAPTER ONE

Amphibian Conservation Status

The decline of amphibian populations began as early as the 1970s and was first made aware to members of herpetology societies from around the world during the First World Congress of Herpetology (1989). Since that initial recognition, subsequent research has shown the severity of the declines with more than one third of extant amphibian species listed in the IUCN Red List Categories of Vulnerable, Endangered or Critically Endangered (IUCN 2017). The most likely threats include anthropogenic land use changes leading to habitat destruction and fragmentation, infection by *Batrachochytrium dendrobatidis*, climate change, overexploitation, environmental chemical pollutants, and introduction of alien species (Blaustein and Kiesecker 2002; Beebee and Griffiths 2005).

The newts and salamanders (Caudata) are the most endangered within the amphibian group, with 47 % of those species classified as threatened (Stuart et al. 2004). Nearly as concerning are the population trends of those species not considered at risk. In the United States, amphibian species listed as Least Concern under the IUCN, showed a mean annual trend of -2.7% from 2002 to 2011 (Adams et al. 2013). While these species of Least Concern show generally stable populations over most of their range, isolated areas of population declines may be early indicators of trouble. For example, the Great

Crested Newt (*Triturus cristatus*) of Europe has seen a drastic reduction in numbers in the second half of the 20th century, despite its relatively wide distribution area. This decline has been primarily attributed to a shortage of suitable bodies of water because of increased agriculture (Langton et al. 2001). Similarly, the North American newt species, *Notophthalmus viridescens* is currently listed as being of Least Concern while one of its subspecies, N. *v. louisianensis* (central newt), is listed as threatened in outermost limits of the species' range in Iowa and Kansas (Whitmore et al. 2013); the loss in numbers is attributed to habitat loss and fragmentation (Camper 1988).

The decline of amphibians has left conservationists struggling to find mitigation measures to prevent further losses. These efforts are complicated because declines are not isolated to degraded landscapes with populations in protected declining at similar rates (Adams et al. 2013). To effectively respond, researchers must first distinguish whether a population decline can be attributed to anthropogenic causes or is part of the natural life history of the species (Băncilă et al. 2010). An accurate assessment of the status of an amphibian species in a geographic area cannot be evaluated from a single snapshot in time. Geographic ranges are reflections of both the current climate and landscapes of the areas, but also remnants of prehistoric range shifts and expansions. Similarly, the distribution of populations within a geographic range is subject to smaller scale changes, both natural and anthropogenic.

Amphibian Population Structure

All North American amphibian distributions can be traced to the climactic fluctuations found during the Pleistocene. Amphibians are highly attuned to changes in

temperature and moisture due to their permeable skin, biphasic lifecycles and unshelled eggs, (Carey and Alexander 2003). As such, the distribution of amphibians at the Last Glacial Maximum (LGM) was very different from present day. This was especially true of temperate regions where climate and landscape changes were most evident. As temperatures warmed from the LGM to present, those populations isolated to refugia were able to expand (Waltari et al. 2007). These migrations are thought to have led to the modern disjunct populations of some amphibians, which can be observed from their current genetic differentiation (Church et al. 2003). However, the lack of fossil data from eastern North America from the Late Tertiary and Early to Middle Pleistocene has left much uncertainty (Graham 1999) and conflicting hypotheses for the origins of disjunct montane and coastal plain populations (Church et al. 2003). Understanding the distributional responses to past climate fluxes can help predict the changes expected from current climate change. The pace at which newly available habitats were historically recolonized can help identify those species unable to relocate fast enough to escape the ongoing changing climate. The current level of isolation and the demographic history can elucidate the ability of species to adapt to disruptive climate changes. Species that were able to maintain gene flow between populations as environmental conditions changed were able to slow the process of geographic differentiation which is now evident in the lack of a genetically divergent population structure. The effects of these historic expansions and contractions are evident in the current population genetic diversity and phylogeographic divergence.

Since amphibians typically have low dispersal capacities and high site fidelity, they often show distinct population genetic structures over even relatively short geographical distances (Duellman and Trueb 1986). Numerous divergent lineages were found for *Pseudacris crucifer*, most likely the result of expansion from southern Appalachian refugia beginning in the late Pliocene. The incongruent levels of diversification revealed in comparative phylogeographic studies have been tentatively linked to dispersal ability (Austin et al. 2004). Inferences about historical dispersal rates can be made from the level of phylogeographic divergence (Avise JC 2000).

Conversely, identification of population dynamics and life history strategies can lead to inference of an expected genetic structure (Neville et al. 2006). Although individual amphibians have a lower dispersal capability than a large mammal or bird, it is possible for the connectivity to be maintained over a large geographic distance if suitable habitat is contiguous. For amphibians this means that each breeding site is within the dispersal distance to another site. Connectivity can be maintained indefinitely as long as no barriers between breeding sites exist and the intervening habitat is favorable. This arrangement of connected populations forming a larger regional population has been identified as a metapopulation. Although the classical metapopulation structure may be true for many amphibian species, for others the characterization may be inaccurate and far more complicated. The specific population structure can be inferred from the genetic structure of the population (Smith and Green 2005). In reality, amphibian populations might not be easily classified by standard population models and may instead show distribution patterns with features from multiple models dependent on characteristics

unique to the species, such a site fidelity, vagility and disturbance tolerance. Metapopulation studies of even the same species have led to different conclusions, indicating that population structure may be adaptive to landscape conditions (Smith and Green 2005; Kinkead et al. 2007). Rowe et al. (2000) found that the natterjack toad (*Bufo calamita*) most likely exhibited a combination of features from population models with either multiple highly connected subpopulations or a mainland island model (Rowe et al. 2000).

When considering metapopulations at the local scale each deme can be viewed as a small population, susceptible to genetic drift. However, since gene flow is the countering force to genetic drift, the between patch dynamics of a metapopulation are central to the genetic makeup at both the local and regional scale. Assessing the genetic diversity of a metapopulation is complex and requires consideration of both spatial and temporal scales to evaluate the genetic size. Completely isolated populations will show the predicted decline in heterozygosity attributed to genetic drift but the fixation of different alleles in each population is entirely random. Therefore, heterozygosity in the global metapopulation will not decline even as individual populations show losses. Conversely, in a metapopulation showing nearly panmixic gene flow rates, total population heterozygosity will decline at the same rate as the subpopulations. As all subpopulations become fixed for the same allele, total population heterozygosity will become zero (Allendorf 2013). Intermediate gene flow is expected to maintain higher levels of heterozygosity both for the total population and subpopulations.

Metapopulation-like populations that deviate from the classical metapopulation present a more complicated picture. It is unlikely that all demes are equivalent in their extinction risk and therefore gene flow may be unilateral with larger stable populations consistently acting as a source for the habitat patches of small extinction prone populations (Gaggiotti and Smouse 1996). The genetic implications of this type of metapopulation structure will vary greatly from the classical model, primarily due to the rate of patch extinction and the characteristics of the founders (Hedrick and Gilpin 1997). Regarding the founders, the two extreme possibilities are that all founders come from the same founding population or that colonization can come randomly from any of the demes. Of these two scenarios the former will reduce the effective population size (N_e) for the total population as will high rates of patch extinction (Slatkin 1977).

Since patterns of population differentiation are most likely dependent on the scale of observation, researchers should examine subpopulation connectivity across a wide range of distances. Using a hierarchical design with inter-population distances ranging from 1–670 km Monsen and Blouin (2004) found that the cascades frog (*Rana cascadae*) showed increased overall genetic differentiation by distance and notably detected a sharp drop in gene flow at 10 km. In general, most studies of large-scale amphibian populations found high levels of spatial structure (Shaffer et al. 2000).

Since the persistence of a metapopulation is dependent on the equilibrium of patch extinction and colonization, the between patch dynamics affecting gene flow are of key importance to understanding genetic divergence of demes (Knaepkens et al. 2004). While it is assumed that variations in species vagility, site fidelity and usage of matrix

composition are among the many natural factors influencing connectivity of habitat patches (Vandewege et al. 2013), alterations in either spatial arrangement or matrix functionality can reduce or eliminate between population immigration. Land use changes that increase inter-patch distances are presumed to be disruptive to amphibians due to their purported limited dispersal abilities (Hale et al. 2013). If the dispersal distance between patches is greater than the species potential maximum migration, then gene flow cannot be expected; however, this distance appears to have been reportedly under-estimated. Although a distance of 1km is frequently used as the threshold for population isolation, a review of the literature found that the average maximum distance moved by anurans and salamanders to be more than double this value (Smith and Green 2005). By accurately calculating the maximum long-distance dispersal for each species, the feasibility of movement between patches can be better assessed.

Determination of the maximum dispersal capability of a species is only one part of understanding the spatial patterns of metapopulation dynamics at the regional level. By incorporating landscape genetics into investigations of population structure and gene flow, a more complete analysis of geographic patterns of genetic variation can be made. A growing body of evidence suggests that amphibians are particularly sensitive to habitat alterations adjacent to breeding populations (Reh and Seitz 1990; Hitchings and Beebee 1997; Rowe et al. 2000; Lampert et al. 2003; Hale et al. 2013). Thus, it is important to consider functional connectivity as well as spatial separation when assessing metapopulation connectivity. In particular, the effects of urbanization have been found to disrupt demographic dispersal rates and the associated gene flow (Reh and Seitz 1990;

Hitchings and Beebee 1997; Vos et al. 2001; Arens et al. 2006; Cushman 2006; Hale et al. 2013). Urbanization can directly impede movement due to the construction of physical barriers or the degradation of habitat such as loss of wetlands and significant increases in light and noise pollution (Eigenbrod et al. 2009). Additionally, the success rate for those individuals that do cross into inhospitable habitat can be very low. A study of six amphibian species in Denmark found that the probability of a successful road crossing was as low as 0.02 (Hels and Buchwald 2001). The implications for anthropogenic habitat fragmentation vary dependent on the specific population dynamics and disturbance tolerance of individual species. Given the degree of habitat fragmentation found in many amphibian ranges, previously connected patches could ultimately be cut off from the regional gene pool. Although the precursor to metapopulation formation is habitat fragmentation, the rate and degree of anthropogenic habitat alteration may not allow for the development of stable metapopulation dynamics, instead resulting in small completely isolated populations with the resulting detrimental declines in genetic variation (Vos et al. 2001). Since variation has been found in both the distance and disturbance tolerance of amphibians to dispersal barriers conservationist are forced to make land conservation decisions based on sometimes arbitrary standards applied to all amphibians (Monsen and Blouin 2004).

If indeed amphibians have experienced the loss of genetic diversity predicted, has there been any evidence of genetic fitness correlations? Since genetic drift and inbreeding expected in small populations have been linked to reductions in fitness characteristics, such as lower survival and decreases in reproductive success, these effects

should be evident in amphibian populations of reduced genetic variation (Samollow and Soulé 1983; Pierce and Mitton 1982; Rowe and Beebee 2005; Johansson et al. 2007). The mechanism behind this reduction in fitness is an increase in autozygosity of deleterious recessive alleles. While lethal alleles are typically selected against and purged from the gene pool, sub-lethal alleles may provide a greater contribution to inbreeding depression. Heterozygosity is linked to overdominance, the greater fitness of hybrid individuals, and bestows greater environmental adaptive potential. Therefore, the loss of heterozygosity should be associated with less fit populations. Hitchings and Beebee (1997) not only showed a correlation between genetic diversity and larval survival in *Bufo bufo* but also linked that reduction in diversity to isolation in urban habitat. Pearman and Garner (2005) found that genetic diversity was strongly associated with larval resistance to mortality following exposure to Ranavirus. Similarly, Bridges et al. (2001) found a link between reduced genetic diversity and insecticide tolerance in southern leopard frogs (*Lithobates sphenocephalus*) attributed to a restriction in migration Given the wide variety of stressors to which amphibians are exposed, e.g. pesticides, climate change and pathogens, genetic adaptability may be the key to survival. Conservation efforts should therefore strive to maintain high levels of genetic diversity in populations. To counter the natural tendency of small populations to lose variation, preservation of gene flow must be maintained.

Small populations that do not meet the criteria of an idealized population such as random mating, constant breeding population size and an equal number of offspring per breeding adult are at greater risk of genetic loss if they become isolated (Kliman et al.

2008). In general, amphibian populations do not meet these assumptions of an idealized population and are known to have very low effective population sizes. Few individuals from the population contribute to the gene pool in a breeding season. They have variable reproductive success, with a single good year interspersed among years of low or no reproductive output (Richter et al. 2003), fluctuating population sizes and skewed breeding sex ratios (Madsen and Loman 2010). Since effective population size is positively correlated with genetic diversity, amphibians with their reported low effective populations are susceptible to low levels of genetic diversity. Pond breeding amphibians share general life-history traits such as low vagility, strong breeding site fidelity, risk of desiccation during dispersal that can limit gene flow. These features can result in highly divided populations and metapopulation-like spatial distribution and dynamics (Semlitsch 1985; Blaustein et al. 1994; Funk et al. 1999; Marsh and Trenham 2001; Palo et al. 2004).

The validity of the classical metapopulation pattern has not been empirically substantiated in amphibians and instead it is based on life history generalizations. Hypothetically, the application of the metapopulation theory to amphibians makes sense with breeding ponds representing the patches and terrestrial habitat, the matrix. However, population subdivision alone does not equal a metapopulation. A metapopulation is a regional assemblage of plants or animals whose permanence is dependent on the stochastic balance of extinction and colonization of discrete populations in habitat patches linked through dispersal of individuals over non-habitat matrix. (Levins 1969; Hanski 1998). Continuance of the population as a whole is dependent on factors influencing extinction and colonization rates, such as the number of habitat patches, the

probability of a population being able to fill vacant patches and the rates and patterns of migration between patches. The classical or Levins metapopulation definition is even more narrowly defined with the following assumptions: demes are identical in size and behavior with no single subpopulation being large enough to sustain the other populations, dispersal occurs uniformly across the entire region with all patches having an equal opportunity to be found and extinctions of demes are independent and therefore patch dynamics are asynchronous so that all populations do not go extinct simultaneously and all patches are equally connected (Hanski and Gilpin 1997). In a review of relevant empirical works, Harrison (1991) found very few populations meeting the classical metapopulation criteria; instead she identified three more commonly found metapopulation-like configurations (Harrison 1991): the source-sink or mainland-island metapopulation in which recolonization stems from one or more extinction resistant population; patchy populations, which effectively exist as a single extinction resistant population due to high connectivity between patches; and non-equilibrium metapopulations in which subpopulation extinction is not part of the normal population dynamics but is attributed to a species regional decline.

Notophthalmus viridescens viridescens

The red-spotted newt (*Notophthlamus viridescens viridescens*) is the second most widely distributed salamander in the United States (Petranka 2010). The red-spotted newt is one of four subspecies of the eastern newt (*Notophthlamus viridescens*) distinguished by dorsal color patterns and geographic range. The three other rare subspecies consist of the broken-striped newt (*N. v. dorsalis*), the central newt (*N. v. louisianensis*), and the

peninsula newt (*N. v. piaropicola*) (Petranka, 2010). Interestingly, an allozyme based study preliminarily indicated that the level of divergence among the four morphs is not great enough to warrant subspecies classification (Gabor and Nice 2004). A re-evaluation of these findings was conducted using the mitochondrial sequences of the ND2 and flanking tRNA-met genes (Lawson and Kilpatrick 2014). Again, grouping by taxonomic designation was not observed; rather, the partitioning of two haplotype groups was determined to be by geographic location. Lawson and Kilpatrick (2014) hypothesized that origination of these genetically distinct groups dates back to the last glacial maximum.

N. v. viridescens is the most widespread of the variants with a range including the Canadian provinces (except western Ontario) and the eastern United States west to central Michigan, central Indiana, Kentucky and Tennessee east of the Mississippi, south to central Georgia and Alabama and northern North Carolina (Collins and Conant 1998). Currently the species is listed by the IUCN as being of Least Concern (IUCN SSC Amphibian Specialist Group 2014) However, according to the IUCN there is a lack of research and monitoring of population trends.

N. v. viridescens has one of the most variable and complex life cycles found in amphibians with several possible morphologically distinct stages including: embryos, larvae, terrestrial efts and lunged adults; aquatic juveniles and adults with lungs; and aquatic adults with gills (Petranka 2010). A review of the literature has revealed equivalent variability in life history traits such as migration timing to and from the breeding ponds, age at first reproduction, body size and existence of paedomorphosis.

Presumably, this developmental plasticity has enabled the newt to be ubiquitous over a large geographic range, encompassing a wide variety of habitat types and climate.

The life cycle most commonly found consists of four stages including the terrestrial juvenile eft. The eft stage does not occur in all populations, particularly in coastal habitat, where neoteny has been observed instead (Brandon and Bremer 1966). The plasticity in life cycles has led to conjecture as to the link between environmental conditions and adaptive life history strategies. The eft stage or lack thereof has garnered the most debate. The eft stage has been purported to be an escape from intraspecific competition during maturation (Healy 1974; Healy 1975) or as a means of dispersal in a species that would otherwise be limited in its ability for colonization or recolonization of vacant habitat (Gill 1978a; Gill 1978b). Healy (1974) assumed that in populations of only neotenic adults, that the eft stage was selected against in those regions and that these populations more representative of an r selected life history strategy. Neoteny has also been suggested as a mechanism for resource partitioning between age classes in a population under intense competition since neoteny has been observed to be negatively correlated with larval density with the eft stage, (Healy 1974; Harris 1987; Harris et al. 1988).

The preferred habitat of sexually mature adult newts is permanent pools in forest openings but they can be found in a variety of water bodies including both permanent and ephemeral ponds, at the shallow areas of large oligotrophic lakes and in still areas of streams (Collins and Conant 1998). *N. v. viridescens* is not found to persist in habitats of less than 50% forest coverage (Gibbs 1998b). Within the ponds, adults will seek out

open sunny areas and sites with submergent and emergent vegetation (Gates and Thompson 1982). Breeding population sizes have been found to vary greatly with the age of pond being the controlling factor (Gill 1978b). Adults commonly migrate seasonally from the water to land for overwintering and aestivation during the summer. Gill (1978a) observed that the mass movement patterns of adults to hibernacula in the Shenandoah mountains of Virginia in August and September was associated with heavy rains. In deeper water adult newts may remain year-round, residing just under the thermocline in summer months (George et al. 1977) and finding ice free areas during the winter (Pitkin and Tilley 1982; Jiang and Claussen 1992).

While adults are capable of terrestrial movement as evidenced by migrations of 0.4 km migrations between breeding and summer residence ponds, this stage has not been found to be a significant dispersal unit (Gill 1979; Roe and Grayson 2008). Instead, extreme philopatry is exhibited by adults returning in the spring to breeding ponds making the likelihood of the discovery of new breeding ponds slight (Gill, 1979). Migration to breeding pools has been observed in both autumn and spring (Healy 1974; Gill 1979; Massey 1990). In those populations where autumn matings occurs, egg laying does not occur until spring and female sperm storage is presumed (Sever 2006). Egg laying is a lengthy process in which the female can take weeks to deposit between 200 and 375 eggs individually wrapped in vegetation (Morin 1983). Incubation lasts 20-35 days depending on water temperature (Bishop 1941).

The length of the larval stage and size at metamorphosis again is variable, depending on geographic range, type of water bodies, larval concentrations and annual

environmental conditions (Harris et al. 1988). Larvae have an extremely low survival rate and according to the limited research on larvae survivorship, the mortality rate is not associated with the size of the breeding population or the age of the pond (Gill 1978b). No correlating condition could be found to explain the sporadic breeding success of a pond nor the frequent failures. In a three-year mark and recapture study Gill (1978b) found that adult losses surpassed juvenile recruitment at most ponds and concluded that recruitment of transforming efts from other ponds prevented local extinctions. Additionally, he found that the ponds all received migrants from one very successful pond, indicating the existence of a source-sink metapopulation structure for the duration of his study period.

The persistence of a metapopulation structure is dependent on adequate gene-flow between patches. If Gill's assessment of newt population structure is accurate, the mechanism for dispersal is most likely the eft stage. Limited and conflicting data exist for this terrestrial portion of the life cycle. Studies predating 1950 estimated the length of the time in the eft stage to be 2 – 4 years (Gage, 1891; Pope 1924; Bishop 1941). Subsequent studies have upwardly revised the time of this terrestrial stage to 3-7 years (Healy 1974, Gill 1978a). Reported eft dispersal distances are varied and inconsistent. Given the small number of studies, the difficulty in recapturing efts and the variation in geographic region and habitat type the inconsistency is not unexpected. Several capture–recapture studies have shown that terrestrial efts are able to travel long distances (Twitty et al. 1966; Johnson 2003). However, Healy (1975), only documented distances of 13m between captures and concluded that efts did not lead a nomadic lifestyle. Instead he

maintained that they form home territories; in his study of Massachusetts efts', home ranges averaged 270m^2. Using fluorescent powder tracking Roe and Grayson (2008) found the maximum distance traveled by a newt in one night to be nearly 80 m. They found that the terrestrial movement patterns of efts were less direct than those of adults, perhaps indicating foraging behavior. The efts often climbed up logs and ferns in a behavior typical of foraging woodland salamanders (Jaeger 1978). In general, all studies reported that eft activity was affected by humidity and rainfall.

All aquatic stages of *N. v. viridescens* are non-selective carnivores, feeding on prey in the same proportions as its abundance (Pitkin and Tilley 1982; Morin 1983; Wilbur and Fauth 1990). Because of their opportunistic feeding strategy, adult red-spotted newts have been found to influence the relative abundance of prey populations such as zooplankton, insects and amphibians and as such are considered keystone species (Morin 1983; Fauth and Resetarits 1991). In communities with potentially dominant species, newt predation has been found to keep such species in check, even in the case of introduced invasive species (Morin 1981; Wilbur et al. 1983; Smith 2006). Smith (2006) found that the presence of *N. viridescens* in experimental ponds reduced the effects of the non-native treefrog, *Osteopilus septentrionalis*. In ponds without *N. viridescens* the presence of *O. septentrionalis* tadpoles was correlated with decreased survival of native species.

Although mark and recapture techniques have been useful to assess demographic connectivity between populations of amphibians, advances in genetic analysis technology have allowed scientists to infer population structure more effectively. Genetic analysis

benefits conservation scientists by providing information on the contemporary changes in genetic composition of populations and by predicting the effects of future land use changes (Manel et al. 2003; Wan et al. 2004) . Landscape genetics quantifies the effects of land use variables and matrix quality on the connectivity of populations using both genetic analysis and GIS tools (Manel et al. 2003). Four biochemical studies of *N. v. viridescens* used electrophoretic analysis of isozymes to assess population genetic structure. Tabachnick (1977) found there was some correlation between genetic differentiation and environmental factors. He did not find significant differentiation between subspecies within *N. v. viridescens*. Merritt et al. (1984) found much higher than expected levels of heterozygosity compared to other amphibian species. He speculated that this higher-than-expected diversity could be the consequence of large effective population sizes or heterogeneous environments. Alternatively, Reilly (1990) found lower levels of genetic diversity than expected for a species with such a wide range. Contradictory to other studies he found *N. v. meridionalis* and *N. v. perstriatus* to differ genetically from *N. v. viredescens.* Gabor and Nice (2004) found that populations of *N. viridescens* clustered by geographic locale not subspecies designation.

The development of genetic markers better tailored to population structure studies have further elucidated the genetic structure of *N. viridescens.* A review of these studies is presented in Chapter Three.

Conservation

Delineation of the biological conservation unit should be a fundamental first step to any conservation effort. Resolving the role a species' life history has contributed to its

phylogeographic history can provide scale for habitat protection. Those species whose long-term survival is dependent on their dispersal capacity are especially vulnerable to anthropogenic habitat fragmentation. Establishing the level and type of habitat disturbances that can be expected to hinder dispersal can help to make landscape-scale protection or mitigation decisions.

For species that presumably exist as a metapopulation, like the red-spotted newt, the extinction and re-colonization dynamics are central to its long-term survival. The shifting source-sink metapopulation structure identified by Gill (1978) is especially vulnerable. Most of the ponds he surveyed had negative population growth (i.e., $r < 0$) and without immigration would have gone extinct. Sjogren (1991) showed that the likelihood of extinction of a metapopulation patch increased by either obvious deterministic causes or by distance from an occupied patch. Because Gill found that the identity of source ponds constantly shifted, loss of habitat connectivity by even one pond could eliminate the rescue-effect for the entire metapopulation. Therefore, it is important to identify how the gene flow rates between breeding ponds are impacted by the matrix of terrestrial habitat. Sampling at various disturbance levels and distances will provide the clearest understanding of the metapopulation dynamics of *N. v. viridescens.* Effective management of a species necessitates this base knowledge in order to maintain or restore lost habitat and dispersal routes (Hanski et al. 1996). While the conservation status of *N. v. viridescens* is currently not of concern, the status of its closely related subspecies relatives may serve as a warning. Striped newts have shown declining numbers since they were first described in 1941, either disappearing from previously occupied sites

altogether or are found in lower population numbers. Habitat fragmentation, as a result of the loss of the native longleaf pine-wiregrass ecosystem and fire suppression, is the purported cause for these losses (Johnson 2005).

CHAPTER TWO

Introduction

Understanding the past and present patterns of gene flow is crucial to conservation efforts, particularly those addressing climate change. In order for a species to persist in a changing landscape it must be able to maintain levels of gene flow to counter the effect of genetic drift in populations. Researchers assessing the genetic diversity and differentiation among populations most often focus on the interaction of gene flow and genetic drift. Through investigation of population connectivity and genetic structure a stronger understanding can be made of the influence a species' life history strategies (Taylor and Hellberg 2003) and dispersal patterns (Webster et al. 2002) make on its long-term population persistence. Assessing the level of genetic connectivity between populations in a minimally altered landscape can provide this base knowledge to better guide conservation efforts.

It is well established that habitat fragmentation is a significant driver of amphibian population declines (Kiesecker et al. 2001; Cushman 2006). The impact and the mitigating strategies vary depending on the population dynamics of a species. These population interactions occur on broad scale of connectivity ranging from a single panmictic population (Wright 1931), to distinct populations highly connected through mutual gene flow (Kimura and Weiss 1964) to metapopulations, where patches of regional populations experience asynchronous re-colonization and extinction (Hanski and Gilpin 1997). The genetic structure associated with each type is reflective of

connectivity and can be used as a diagnostic tool to determine the population dynamics. Panmictic populations are associated with low levels of genetic differentiation in combination with high levels of genetic diversity among populations (Wright 1943). Discrete populations linked through high levels of gene flow show an increase in genetic differentiation as geographic distance increases between populations (Hutchison and Templeton 1999). For metapopulations, the genetic structure is dependent on how the re-colonization of patches occurs. If only a few populations provide low numbers of individuals, then one would expect genetic differentiation between populations to be high with low within population genetic diversity. The gene pool of each population would consist of genes contributed by a few individuals, similar to that of the founder's effect (Wade and McCauley 1988). Conversely, if there are many populations contributing large numbers of individuals then differentiation among populations will be low and genetic diversity within the populations will be high. According to Gill (1978b) the red-spotted newt population exists as a metapopulation in which the majority of subpopulations are reproductive failures most of the time. In his three-year study he found that only one pond consistently had reproductive output rates at or above the replacement rate. He concluded that the other ponds in the study area were supported by immigration. Since adult newts were found to show strong site fidelity, the eft stage was presumed to be the dispersal stage. This metapopulation structure is characterized by many sink populations dependent on a single source pond with a very small effective population size. Given the non-perpetuating nature of the ponds, the regional dynamics are central to the persistence of the population as a whole. Depending on the dispersal

capability of the long-lived eft stage, the geographic expanse of the regional population might only be limited by unfavorable matrix habitat. The result would be an intrametapopulational panmixis extending as far as the suitable habitat remained unfragmented. As such, genetic continuity would be maintained among all pond populations, eliminating deme integrity and any local differentiation (Wright 1931; Levins 1970).

For this study I assessed the population structure of the red-spotted newt in an expanded geographic area of Gill's study (1978a). Through the use of genetic sequences of mtDNA, I was able to determine the level of differentiation between these adjacent ponds and a pond isolated by distance and anthropogenic disturbance.

Study Sites and Methods

Study Sites

Red-spotted newts were collected at 11 ponds in Virginia over the course of this study under Virginia Department of Game and Inland Fisheries (VDGIF) Scientific Collection permit 053953. Field methods regarding newt collection were approved by the George Mason University Institutional Animal Care and Use Committee (IACUC).

The study sites are distributed between two mountain ranges in Virginia (Figure 1). Seven of the ponds are located in the Shenandoah Mountains of Rockingham County, Virginia and two are located in the Massanutten Mountains of Shenandoah County. The Rockingham County and Massanutten Mountain study areas are separated by the

Shenandoah Valley approximately 60 miles apart. Unlike the adjacent mountain ranges the valley has numerous natural ponds ranging in size from less than 0.04 ha to more than 1.5 ha. This collection of ponds, known as the Shenandoah Valley Sinkhole Pond (SVSP) system was formed during the Pleistocene. A weak carbonate limestone stratum under a layer of compacted sand, silt and clay resulted in the formation of sinkholes. The clay deposits created impermeable layers in some of the sinkholes which filled with surface runoff or ground water dependent on the hydrology (Fleming and VanAlstine 1999).

From its initial settlement by Europeans in the 1730s, the Shenandoah Valley was converted to some of the highest producing agricultural areas in Virginia, unlike the adjacent mountains which were spared from conversion because of their rugged terrain and poorer soils. This long modern history of deforestation led to the impression that the valley was open prairie before the arrival of colonists and that it had been maintained by native populations for hunting (Shaler 1891). This is no longer widely accepted, but not entirely disproved (Kercheval 1925). In the eastern United States a maximum of deforestation attributed to agriculture peaked in the late-nineteenth or early twentieth century (Hart 2003). While reforestation of agricultural areas was seen in much of Virginia in the late twentieth century, that was not the case in the Shenandoah Valley. In 1997, Augusta and Rockingham counties were ranked economically as the top two agricultural counties in Virginia (Virginia Agricultural Statistics Service 2000). Although the Shenandoah Valley maintains its rural character, land use began to shift from agriculture to suburban housing developments during the late 20th century.

Both the Rockingham County and Massanutten study areas are found within the George Washington National Forest. According to the 2014 Land and Resource Management Plan for the George Washington National Forest the land encompassing the study ponds is designated with the following classifications: Special Biological or Geological Area, Mosaics of Wildlife Habitat, Scenic Corridor and an ATV Area. The closest national forest border to the ponds in the Rockingham study area is 2.4 km on the east of Upper Second Mountain Pond and 11.5 kms to the west of Sweigert pond. Large unfragmented tracts of forest for 100's of kilometers can be found north and south of the Rockingham sites following the range of the Appalachian Mountains. The closest national forest border from the Massanutten study ponds is less than 1.4 km to the west of the ponds. The Massanutten mountains are 80 km in length and only 9.5 km wide. The Massanutten mountain range is shaped like a canoe with Fort Valley making up the center. Fort Valley is not under the protection of George Washington National Forest and mostly consists of deforested, private farmland. The entire Massanutten range is within the Shenandoah Valley.

The Rockingham County ponds were chosen because this geographic area was used in a study of the metapopulation ecology of red-spotted newt by Douglas Gill in the 1970s. Three of the twelve ponds surveyed by Gill were used in this study. The mountain range in which these ponds are found forms the western border of the

Shenandoah Valley and is the eastern-most position on the Appalachian Mountain range; as such it exists in a rain shadow. The Rockingham County study area consists of a series of ridges and short steep-sided valleys with the highest ridge at approximately 1,085 meters. All study ponds are located along adjacent ridge lines. These ponds are all manmade and are the only water bodies located on the mountain range. The age of the ponds ranges from 120 years old (Trespass pond) to 55 years old for the ponds on Second Mountain. All ponds are roughly circular in shape and measure 10 to 14 meters in diameter except for Sweigert pond which measures 55 meters in diameter. The ponds are shallow, at less than 2 meters deep, and vegetated along the shore (Figures 2 and 3). They are all dependent on rainwater as a water source. Prior to the construction of these ponds, few natural ponds existed on this range in the preceding two hundred years. Although the oldest ponds were originally created as watering holes for cattle in open areas, all ponds are now located within the canopy of secondary forests.

The two ponds in the Massanutten mountain range are located below the western ridge adjacent to Shenandoah Valley at an elevation of approximately 450 meters, which is substantially lower than the Rockingham County ponds. The Massanutten ponds are similar in size to the Rockingham County ponds and likewise located within the canopy of secondary, mature forests. While both of these ponds are also manmade, natural streams can be found within the Massanutten Mountain range. The Peters Mill Pond (figure 4) is located less than 100 meters from a permanent stream. Spring-fed and limestone creeks can be found flanking both sides of the Massanutten Mountain range.

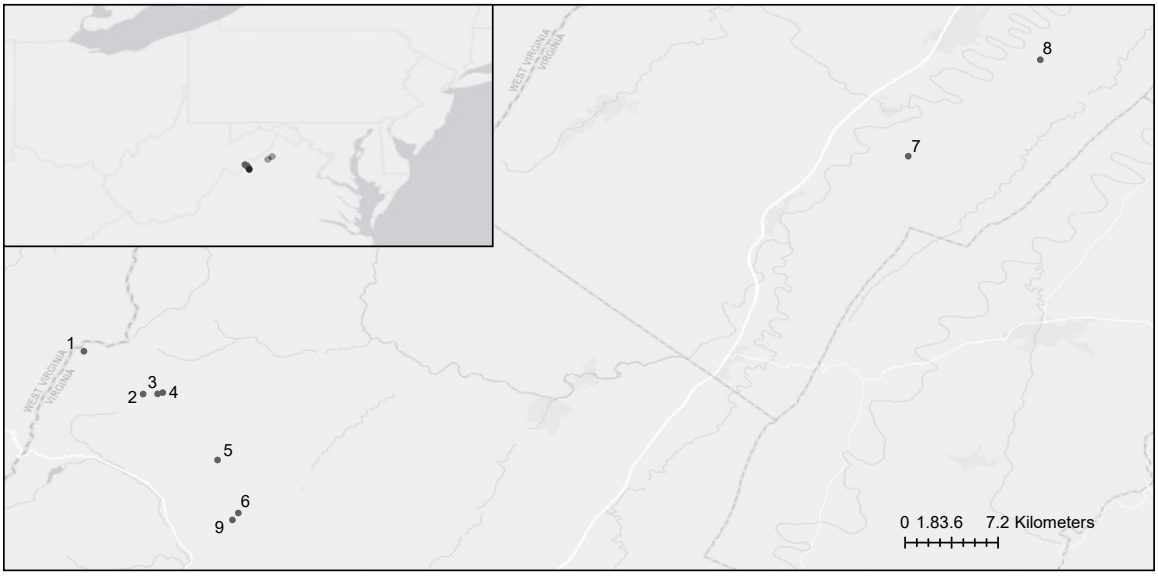

Figure 1 Map of Study Sites

Map of collecting sites. Geographic coordinates and elevation are located on Table 1. (1) Sweigert, (2) Hidden Pond, (3) Trespass Pond, (4) Long Run Road Building, (5) Long Run Road I, (6) Upper Second Mountain, (7) Edinburgh, (8) Peter's Mill, (9) Second Mountain 3

Figure 2 Hidden Pond on Tomahawk Mountain in the Rockingham County study area

Figure 3 Long Run Road I on Pond Ridge in the Rockingham Country study area

Figure 4 Peter's Mill Pond on Powell Mountain in the Massanutten range.

Table 1 Collection sites.

Collection Site	Pond Code	latitude	Longitude	Elevation	Gill's Pond (1978)
Sweigert	SWE	38.6529	-79.1229	1130	
Hidden Pond	HP	38.6231	-79.0817	1149	
Trespass	TP	38.6232	-79.0716	1071	Lower Feedstone
Long Run Road Building	LRRB	38.6241	-79.0681	1083	
Long Run Road I	LRRI	38.5771	-79.0299	1035	White Oak Flat
Upper Second Mountain	U2M	38.5401	-79.0155	905	
Edinburgh	EB	38.7889	-78.5495	489	
Peter's Mill	PM	38.8561	-78.4577	1008	
Second Mountain 3	2M3	38.5353	-79.0196	880	Lower Second Mt II

Sites are listed in order from west to east, except for Second Mountain 3. For this pond only RV-ml sequences were generated, and it is not included in the analysis of the full DNA segment (HDL-F1 to 12S600H).

Methods

609 adult newts were captured from eleven ponds in 2015 and 2016 during the period from June to August. Only one pond located on the Feedstone Camp private property yielded no newts and is not included in the list of surveyed ponds. According to a Feedstone Hunt Club member, this pond had been dredged the previous year. Nine un-baited collapsible mesh minnow traps were placed in equal intervals along the shallow, vegetated shoreline of ponds. Traps were secured by string to nearby trees or rocks to ensure they were not entirely submerged in water (Figure 4). For the first three ponds surveyed, six Dewsbury Newt Traps were also set but were unsuccessful. These traps are used to capture newts found on the bottom of the ponds and are commonly used to capture the Crested Newt. Presumably these traps were not effective with red-spotted newts because they are found in the water column and along the shore. Traps were set between 1500 and 1700 hrs and checked and emptied the next morning between 0900 and 1000 hrs.

Skin swabs were collected from each newt by running the swab ten times on the dorsal side. The use of skin swabs has been found to be an efficient method of DNA collection, both simplifying the process and reducing stress to the animal (Prunier et al. 2012). Swabs were air dried at the ambient temperature and stored in a cooler with ice packs during transport until they could be frozen at -80°C within 24 hours. Because collections were only conducted at each pond once, it was not necessary to mark the newts before they were returned to the pond.

DNA sequences from a subset of nine of the ponds were used in this study. DNA extractions were initially performed using the FastDNA Spin Kit. After the extractions, PCRs using universal primers for 18S and 12S were conducted to verify the presence of DNA. Those samples that showed clear product on an agarose gel for the 12S and 18S product were then used in an attempt to amplify microsatellite sequences from published primers (Croshaw and Glen 2003). After multiple unsuccessful efforts to optimize the PCR to obtain reproducible microsatellite results, I changed the extraction method to the QIAGEN DNeasy Blood and Tissue Kit. Continued issues with the use of microsatellite markers led to the use of mtDNA sequences for this study. Of the samples used for the mitochondrial amplification in this study all but two were extracted using the QIAGEN method. The protocol for the Qiagen kit was changed to accommodate for the use of swabs; the time period for initial digestion with protease at 56° was increased to an hour.

MtDNA was amplified using primers HDL-F1 (GGCACCCAAAGCCARAATT) (Yuichi et al. 2005) and 12S600H (TCGATTATAGAACAGGCTCCTCT) (Zhang et al. 2008) yielding a total length of approximately 1300 bps. This sequence included the D-loop, tRNA-pro, tRNA-phe and part of the 12S rRNA (Figure 5). The PCR was conducted in a total volume of 25 µl containing 2.5 µl of DNA template, 2.5 µl 10× buffer with $MgCl_2$, 1.25 µl forward primer (10 mM), 1.25 µl reverse primer (10 mM), 2.5 µl dNTPs (2 mM each), 2.5 µl BSA (0.01%), 0.2 µl taq polymerase (5 U/µl) and 12.3 µl DEPC water. PCR conditions consisted of an initial cycle at 95°C for 11min, 40 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10

min. PCR products that yielded a clear band on agarose gel by electrophoresis were purified and sequenced using capillary electrophoresis sequencing.

Sequencing was done using three primers HDL-F1 (GGCACCCAAAGCCARAATT), 12S600H (TCGATTATAGAACAGGCTCCTCT) and RV-ml (GAGGGTGTGGCTAAACAAG) (Whitmore et al. 2013). Individual DNA sequences were edited and assembled using Sequencher 5.4.6 software. The assembled sequences were aligned using Geneious 8.0.5.

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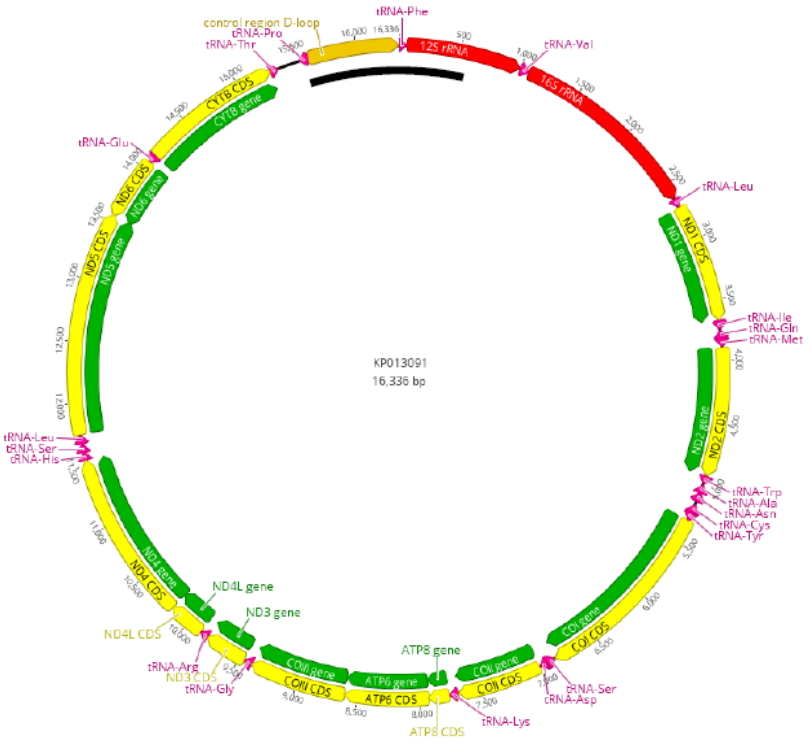

Figure 5 MtDNA fragment location on the mitochondria.

Data Analysis

Analyses were performed on two sets of data. The assembled fragments using all three primers yielded 47 sequences from 8 collection sites. To assess a larger sample size, fragments generated using only the RV-ml primer were analyzed independently, yielding 89 sequences from 9 collection sites. For both sequence data sets calculations of genetic diversity, as indicated by the number of segregating sites (S), number of haplotypes (h), nucleotide diversity (π), haplotype diversity (Hd) and the average number of nucleotide differences (k) were done using DnaSP v6.0. Nucleotide diversity is defined as the average number of nucleotide differences per site in pairwise comparisons of randomly selected sequences in a population. Haplotype diversity is defined as the probability that two randomly sampled alleles differ.

The Arlequin 3.5 software was used to perform an analysis of molecular variance (AMOVA) to analyze the level of genetic differentiation among and within populations. The DNA divergences among populations (F_{ST}) were measured, and the significances were tested using 1023 permutations. Populations were clustered into four groups reflective of the mountain ranges. AMOVA was also performed looking at pairwise differences among and within these groups.

To visualize the relationship among the haplotypes, a haplotype network was generated by means of the Integer Neighbor Joining Network (IntNJ) option in PopART v 1.7. The IntNJ is particularly useful for low-divergence data sets. The tree begins with a neighbor-joining network that was inferred from a distance matrix. Integer edge

lengths calculated by integer linear programming are added to the network (Leigh and Bryant 2015).

Results: Full fragment (HDL-F1, RV-ml and 12S600H)

Diversity Indices

For the concatenated sequence using all three primers (HDL-F1, RV-ml and 12S600H) I analyzed a fragment of 1026 bp of mitochondrial DNA, for a total of 47 individuals from eight wild populations of *N. v. viridescens.* Since the Peters Mill population contained only one sample, it was omitted from any geographical structure analyses. From this data set I identified 13 total segregating sites resulting in 11 haplotypes. Nucleotide diversity was very low at 0.00149 (Table 2). The difference between all haplotypes, except for haplotypes 1 and 2 which were both represented by a single individual each, was a one nucleotide difference from haplotype 11. Haplotype 11 was the most common and was found in all ponds except for the Edinburgh site. It represented 37.5 % of the total data set. Haplotype 7 was only found at the Edinburgh pond and accounted for 57% of the samples from that population. Haplotypes 1 and 10 were also unique to the Edinburgh Pond and were represented by one individual each. Three other haplotypes (2, 3 and 5) were also represented by single individuals in the Rockingham ponds (Table 3). The haplotype diversity for the overall population was moderate Hd = 0.795 but the nucleotide diversity was very low at $\pi = 0.00149$. This combination of measurable

haplotype diversity and low nucleotide diversity can be a sign of rapid demographic expansion from a small effective population size (Avise 2000).

The haplotype network (Figure 6) illustrates the relationship between haplotype 11, the most common, which was found in all but one of the ponds and haplotypes around it. The starlike clustering pattern shows very low levels of sequence divergence and low levels of genetic differentiation. Even though haplotype 11 was not found in the Edinburgh site, haplotype 7, the most common sequence at that location, only differs by a single nucleotide.

Table 2 Diversity indices for populations

Population	*n*	*S*	*h*	*Hd*	*π*	*k*
Sweigert	8	3	4	0.821 ± 0.10	0.00119	1.214
Hidden Pond	3	0	1	0	0	0
Trespass	3	6	3	1 ± 0.272	0.00391	4
Long Run Road Building	6	2	3	0.600 ± 0.215	0.00065	0.667
Long Run Road I	4	2	3	0.833 ± 0.222	0.00098	1
Upper Second Mountain	8	3	4	0.750 ± 0.139	0.00091	0.929
Edinburgh	14	9	4	0.626 ± 0.110	0.00193	1.197
Peters Mill	1					
Rockingham sites	32	10	8	0.696 ± 0.080	0.00115	1.169
All populations	47	13	11	0.795 ± 0.042	0.00149	1.508

12S600H to HDL-F1 fragment information: Sample size (n), number of segregating sites (S), number of haplotypes (h), haplotype diversity (Hd) ≠ SD, nucleotide diversity (π), and average number of nucleotide differences (k) per population

Table 3 Mitochondrial haplotype frequencies by population

Site	Hap 1	Hap 2	Hap 3	Hap 4	Hap 5	Hap 6	Hap 7	Hap 8	Hap 9	Hap 10	Hap 11
SWE	-	-	-	0.25	0.13	-	-	0.25	-	-	0.38
TP	-	0.33	-	0.33	-	-	-	-	-	-	0.33
HP	-	-	-	-	-	-	-	-	-	-	1.00
LRR B	-	-	-	-	-	0.17	-	-	0.17	-	0.67
LRR I	-	-	-	0.25	-	-	-	-	0.25	-	0.50
U2M	-	-	0.13	0.13	-	0.25	-	-	-	-	0.50
EB	0.07	-	-	0.29	-	-	0.57	-	-	0.07	-
PM	-	-	-	-	-	-	-	-	-	-	1.00

Populations are listed in geographic order (west to east). Haplotype (h) numbers correspond to the numbers on the Integer Neighbor Joining network (Figure 7)

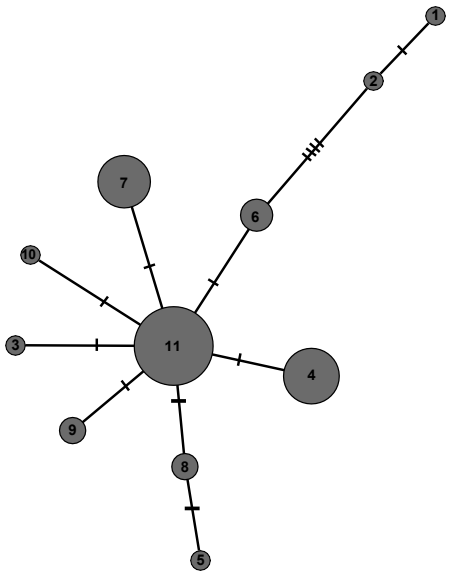

Figure 6 Integer Neighbor Joining network of full fragment haplotypes
Fragments generated using primers 12S600H to HDL-F1 fragment.

Geographical Structure

AMOVA results (Table 4) show that the overall genetic variation within populations (87.16%) was much larger than the variation among populations (12.84%). Pairwise F_{ST} values between populations were only significant between the Edinburgh population and four other populations, Sweigert, Hidden Pond, Long Run Road Building and Upper Second Mountain (Table 5). The Edinburgh population is geographically the furthest removed from the other ponds and is separated by the largely deforested Shenandoah Valley. The AMOVA results grouping ponds by their mountain range location shows that genetic variation between groups only contributed 14.95% of variation; within population variation accounted for 86.00%. The F_{ST} values for ungrouped populations (0.12835) indicates very low differentiation. F_{SC} and F_{CT} values which indicated differentiation among populations between groups and between groups, respectively were not significant.

The geographic distribution of haplotypes shows that haplotype 11, the most common, was found at all of the ponds in the Rockingham County study sites. It was not found at all in the Edinburgh pond located in the Massanutten range. Edinburgh pond had three unique haplotypes not found in any of the Rockingham County sites (Figure 7) However, these haplotypes only differ by one nucleotide from haplotype 11.

Table 4 AMOVA Results

Groups of samples	Source of variation	d.f.	Variance components	Variation (%)	Fixation index
All (ungrouped)	Among populations	6	0.146	12.84	
	Within populations	39	0.991	87.6	
	Total	45	1.1371		F_{ST} = 0.128*
Grouped	Between groups	3	0.1727	14.95	F_{CT} = 0.149
	Between populations within groups	3	-0.1092	-0.95	F_{SC} = -0.011
	Within populations	39	1.152	86	F_{ST} = 0.140*

F_{ST} fixation index within populations; F_{SC} fixation index among populations within groups; F_{CT} fixation index between groups. *P < 0.015. Ponds were grouped by their mountain range location.

Table 5 Distance method: Population pairwise F_{ST}s (12S600H to HDL-F1 fragment)

	SWE	TP	HP	LRRB	LRRI	U2M	EB	
SWE	0							*
TP	0.04721	0						
HP	0.14721	0.11111	0					*
LRRB	0.07336	0.09091	-0.05882	0				*
LRRI	-0.05923	-0.05028	0.07692	-0.13305	0			
U2M	0.03448	0.04481	0.06145	-0.08419	-0.09492	0		*
EB	0.16678	0.09944	0.33353	0.25051	0.12689	0.19091	0	

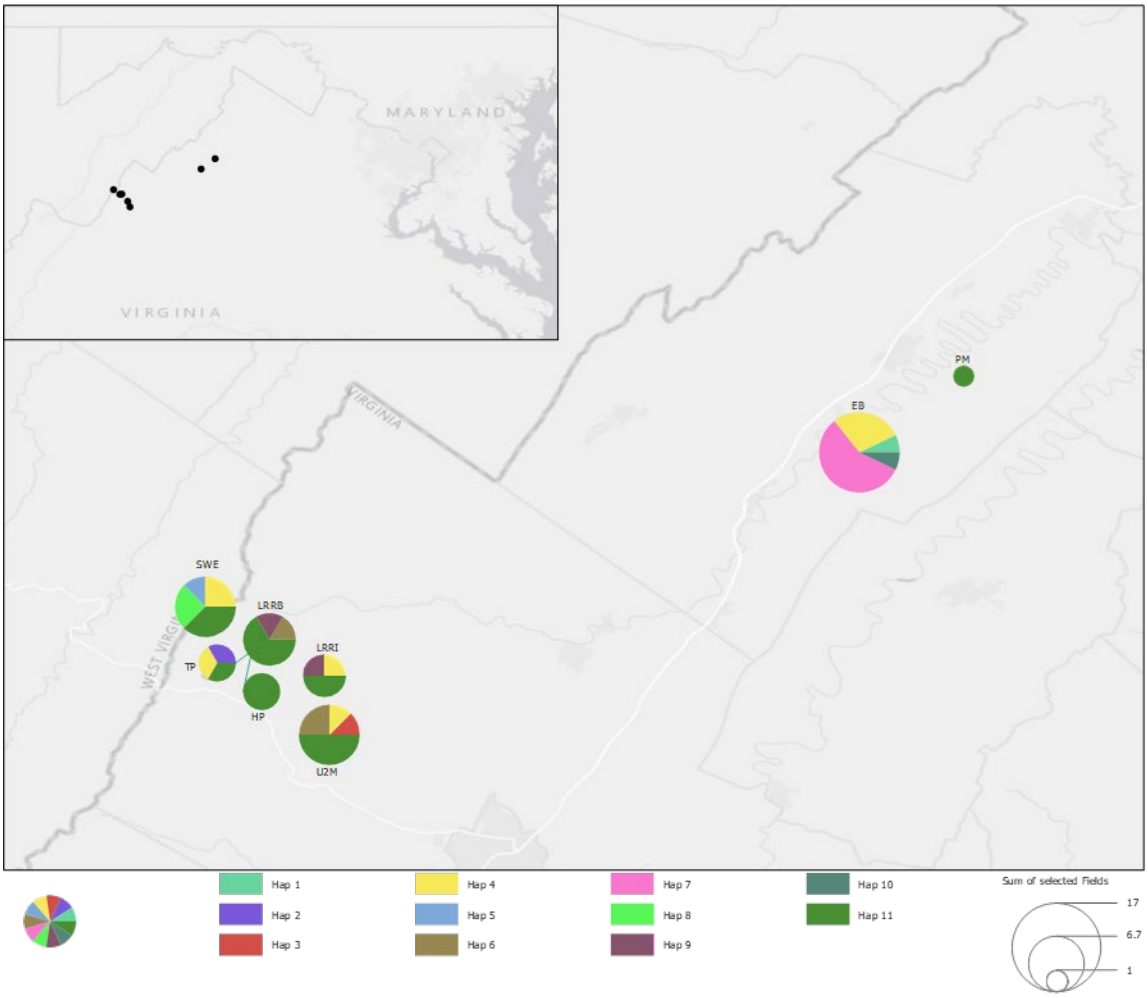

Figure 7 Distribution of haplotype groups by locality (12S600H to HDL-F1 fragment).

Results RV-ml fragments

Diversity Indices

In an effort to enhance the information gained using the data set from the full sequence I used a shorter sequence generated using just the RV-ml primer. The sample size was increased to 89 individuals collected from nine ponds. The resulting 269 bp sequence did not further the understanding of the population structure beyond the information gained from the full fragment data set and data from the geographical analysis is not presented here. Only 5 haplotypes were identified with 3 segregating sites. The nucleotide diversity was extremely low ($\pi = 0.00183$) (Table 6). The F_{ST} value was insignificant ($p = 0.409$) and as such the population pairwise F_{ST}s showed no differentiation between populations. Haplotypes 1 and 2 made up 92% of the total population (Table 7). No haplotype was more than a single nucleotide difference from haplotype 1 or 2, as seen in the Integer Neighbor Joining network (Figure 9).

Table 6 Diversity indices for population (RV-ml frag)

Population	N	S	h	Hd	π	k
Sweigert	18	2	3	0.621	0.00273	0.719
Hidden Pond	5	1	2	0.400	0.00145	0.00145
Trespass	4	2	3	1.000	0.00558	1.500
Long Run Road Building	7	1	2	0.286	0.00104	0.286
Long Run Road I	11	1	2	0.436	0.436	0.436
Upper Second Mountain	8	1	2	0.250	0.00092	0.25
Second Mountain Three	2	1	2	1.000	0.00364	1.00
Edinburgh	26	2	3	0.428	0.00168	0.446
Peters Mill	8	2	2	0.250	0.00183	0.500
All populations	89	3	5	0.420	0.00183	0.477

RV-ml fragment sequence: Sample size (n), number of segregating sites (S), number of haplotypes (h), haplotype diversity (Hd) ≠ SD, nucleotide diversity (π), and average number of nucleotide differences (k) per population

Table 7 RV-ml Fragment haplotype frequencies by population

Site	Hap 1	Hap 2	Hap 3	Hap 4	Hap 5
SWE	0.56	0.28	-	-	0.17
HP	0.80	-	0.20	-	-
TP	0.50	0.25	0.25	-	-
LRRB	1.00	-	-	-	-
LRRI	0.73	0.27	-	-	-
U2M	0.88	0.13	-	-	-
2M3	1.00	-	-	-	-
EB	0.73	0.23	0.04	-	-
PM	0.88	-	-	0.13	-

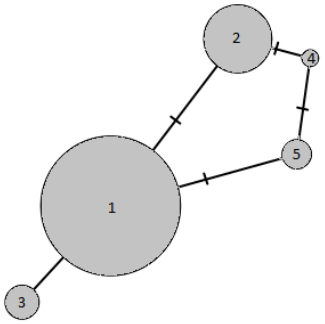

Figure 8 RV-ml fragment Integer Neighbor Joining network

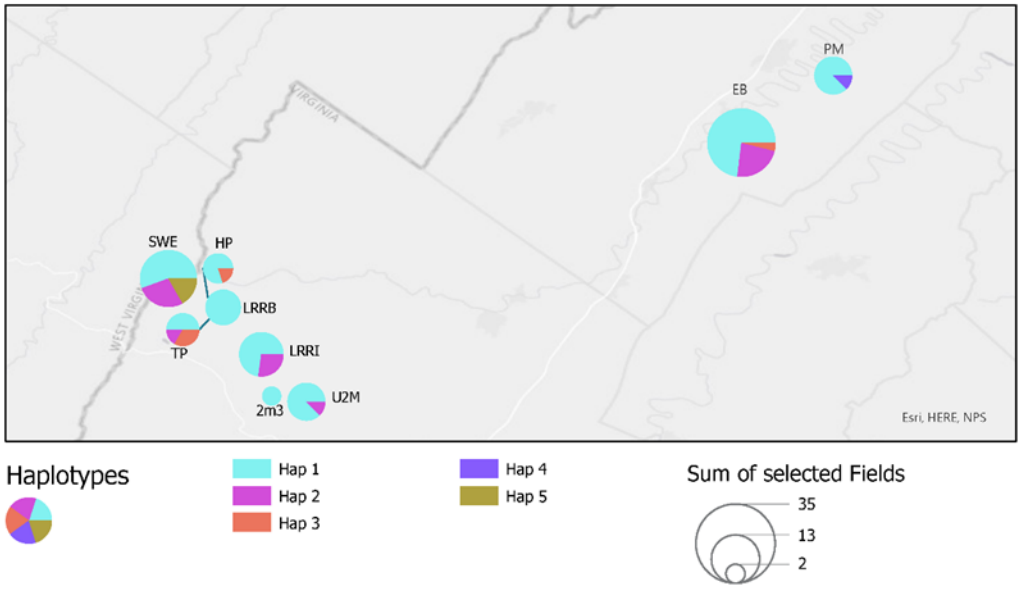

Figure 9 Distribution of haplotype groups by locality (RV-ml fragment).

Discussion

My study supports the hypothesis that the red-spotted newt is highly dispersive and capable of maintaining genetic connectivity between breeding sites over a large geographic area despite potential topographic barriers. As such, no pattern of genetic differentiation was found between populations on adjacent ridges in the same mountain range. Sufficient gene flow between populations can reduce or eliminate the process of geographic differential and has been observed in large, mobile animals (Vila et al. 2003). These findings are incongruent with the observed tendency for amphibians to maintain genetic differentiation over relatively short distances (Beebee 1996). The limited dispersal capacity of most amphibians has been attributed to a pattern of disconnected populations. Ridgelines have been shown to prevent gene flow in amphibians (Funk et al. 2005; Murphy et al. 2010) and salamander populations were found to be fragmented by dry grassland habitat (Rittenhouse & Semlitsch 2006).

In a study in 1975 of newts conducted in the same geographic area as this study, Douglas Gill concluded that the population structure was that of a metapopulation. He found that individual breeding ponds only sporadically had reproductive rates high enough to persist long-term without immigration. From the persistence of newt populations at these reproductive sinks, he inferred that the breeding adults at these ponds were replaced by immigrants, not their own progeny. Furthermore, he observed rapid colonization of newly created ponds on the Second Mountain and Gauley Ridge ridges. From these data he surmised that the red-spotted newt is adapted to the shifting, temporary nature of beaver ponds. Based on the newt densities of the ponds he studied,

he concluded that newt populations would reach carrying capacity at a pond in approximately 50 years which is longer than the lifespan of a typical beaver pond.

Despite the geographic distance and the steep topography, populations found in the Rockingham County range show no indication of geographical differentiation. The unfragmented forest coverage provided a terrestrial habitat suitable for eft migration. The Rockingham populations are closely related to each other as indicated by very low and insignificant pairwise F_{ST} values. Within the Rockingham County populations, the total haplotype diversity (Hd) was calculated as 0.696 with a nucleotide diversity (π) of 0.00115. Eight Rockingham haplotypes were identified. However, all but two of the haplotypes were only one nucleotide removed from haplotype 11, the most common type. This is evident from the Integer Neighbor network (Figure 6). An arrangement of one common haplotype with others in lower frequencies or singular haplotypes is a pattern often attributed to populations that have recently gone through a range expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992). Only haplotypes 1 and 2 varied from haplotype 11 by more than one nucleotide change. Each was represented by a single individual. It is possible that an increase in the total sample size could potentially provide a truer representation of haplotypes present and identify missed population structures. However, given the very low nucleotide diversity it is more likely that the lack of genetic diversity between populations can be attributed to a shared demographic history. The unfragmented landscape between the Rockingham sites allowed for unrestricted gene flow. Across a similar geographic distance, Iowa populations of *N. v. l*ouisianensis showed significant genetic divergence. This genetic separation was attributed to

allopatric divergence in the early Pleistocene. Although Iowa had been recolonized by *N. v. l*ouisianensis following the last glacial retreat, remnants of the genetic differentiation between distinct northern and southern populations were still evident. The persistent pattern of isolation may indicate reduced dispersal capacity over unfavorable terrestrial habitat. Significant levels of population isolation were identified between populations only 6 – 7 km apart in the same drainage (Whitmore et al. 2013). Likewise, Johnson (2001) found a high degree of isolation between populations of *N. perstriatus*. This was attributed to patchy suitable habitat restricting long distance dispersal. Both of these studies looked at peripheral populations which are often more genetically distinct than central populations (Lammi et al. 1999). In contrast, the favorable habitat matrix between the Rockingham ponds presumably supported high levels of connectivity and the dilution of any Pleistocene phylogeographic signal.

The lack of evidence for genetic geographic differentiation between the Rockingham County population adds support to Gill's (1978b) assumption of a metapopulation structure. The low nucleotide diversity and lack of geographically structured clades suggest a single panmictic population. Additionally, the presence of newts at every surveyed manmade pond, except for a single recently dredged pond, in a landscape historically without standing water illustrates the dispersal ability of the red-spotted newt. Gill (1978b) found a collection of ponds created just ten years earlier to all contain newts. He concluded that the newt metapopulation structure was adapted to the periodic shifting of beaver ponds. The natural, ephemeral, sinkhole ponds of the Shenandoah Valley prior to deforestation would have provided the ideal habitat for red-

spotted newts. The cover of forest would have allowed dispersal of efts to recolonize new ponds as their natal ponds receded. According to limited fossil evidence, newts have been present in the Shenandoah Valley since the Pleistocene period (Guilday 1962). The last glacial maximum advance about 18,000 years ago during the late Pleistocene was followed by a warming trend 7000 – 5000 years ago. As temperatures warmed and became drier after the last glacial retreat, dispersal into the adjacent mountains would have occurred (Hoffman 1987). The cooler, forested Appalachian Mountain range would have provided a corridor for austral taxa expanding their ranges. Currently these mountains present an unfragmented, contiguous area of forest which allows for nearly unimpeded migration by the red-spotted newt. This is in contrast to the Shenandoah Valley's highly fragmented landscape. Although surveys in the 1980s and 90s found red-spotted newts were common in the Shenandoah Valley ponds (Mitchell and Buhlmann 1999) it is unlikely the dynamics of a metapopulation structure remain intact given the high level of habitat fragmentation.

DNA sequences from only pond outside of the Rockingham field site were analyzed. While any conclusions reached due to this small sample size are speculative, the preliminary results prove interesting and warrant further investigation. Three unique haplotypes were found exclusively in this pond, with haplotype 7 comprising 57% of this population. This was the only pond for which significant, albeit low, pairwise F_{ST} values were found. The very low nucleotide differentiation from the Rockingham populations do not separate the populations into distinct demes but the unique prevalent haplotype at this pond could be an indication of early separation. Edinburg pond is located in the

Massanutten mountain range and separated by Shenandoah Valley approximately 48 kilometers from the nearest Rockingham County. Since post-European settlement the valley has experienced extensive deforestation and conversion to agriculture. There is some evidence that deforestation may have taken place long before the arrival of European settlers and that Native Americans maintained the valley as a prairie through the use of fire (Shaler 1891). Although this is highly speculative, if true it would indicate that anthropogenic habitat fragmentation predated the 200 years of European settlement and industrialization. Developed landcover, i.e., deforestation has been shown to have the greatest negative influence on newt presence (Rinehart et al. 2009). Some studies have indicated that the red-spotted newts avoid forest edge over more interior locations (Anderson 2012; Gibbs 1998a; Gibbs 1998b). Presumably, this avoidance of edge habitat is a result of selective eft migrations. Deforestation has been found to limit dispersal capabilities in salamanders through the reduction in leaf litter depth, increased exposure and alterations in microclimatic conditions. Deforested areas, regardless of pond availability, may be restrictive to newt colonization. Because the red-spotted newt exists as a metapopulation it is particularly vulnerable to population collapse caused by riparian forest fragmentation (Gill 1978b; Gibbs 1998a).

Understanding the historical biogeography of the red-spotted newt can help in the formation of conservation efforts. The findings that the endangered *N. viridescens* subspecies, *N. v. louisianensis* and *N. v. perstriatus* are experiencing genetic differentiation attributed to habitat fragmentation may be a warning for the more common *N. v. viridescens*. While preliminary, the indication that a reduction in gene flow across

the Shenandoah Valley may be contributing to genetic differentiation highlights a need for expanded geographic sampling. Deciphering the complicated phylogeographic history and its relationship to the newt's life history may provide insight about future distribution patterns. These patterns which evolved after the most recent glacial period can help us to understand and predict the responses to current climate changes.

The relative permanence of the Rockingham ponds has afforded a unique opportunity to observe the long-term population structure of a species' metapopulation adapted to the moderate turnover of breeding sites. Several of the ponds I surveyed in this study appeared to be in late succession with large populations of emergent plants, thick layers of decomposing material and a depth of less than a meter. Since no new ponds are currently being constructed in this area, this population of red-spotted newts may represent the full lifespan of a metapopulation. Colonization occurred as these man-made bodies of water were created and high levels of gene flow have maintained a network of linked populations. The inevitable succession from pond to upland habitat will happen unless human intervention occurs. Given the unsuccessful breeding rates of most populations observed by Gill, existing populations may still experience population crashes if source ponds are no longer available. If suitable breeding ponds cannot be accessed, the wanderings of the eft may be in vain. Ultimately habitat fragmentation may lead to a complete regional extinction of this metapopulation (White and Smith 2018)

CHAPTER THREE

Genetic Analysis of *Notophthalmus viridescens*

While the results of genetic studies have helped to elucidate evolutionary relationships between the sub-species and population structure of *N. viridescens*, the small number of studies as well as the inconsistency in the use of genetic markers, limits the ability to make broad conclusions about the species or comparisons between the studies. The studies conducted thus far have focused on the rarer subspecies of *N. viridescens* or on the phylogenetic relationships between the subspecies, rather than the red-spotted newt's population structure alone. Additionally, since population studies were conducted at the periphery of the habitat range, they may not be a true representation of the population structure as a whole (Figure10).

Assessment of phylogeographic relationships through the use of mitochondrial DNA has been a common approach for the last three decades. The geographical distribution of haplotypes can be used to detect past connectivity and provide insight as to the natural processes affecting their distribution. Recently however, the efficacy of mitochondrial markers for small-scale population studies, particularly isolation by distance (IBD), has been questioned in favor of using microsatellites, MHC or most recently Restriction Associated DNA sequencing (RADseq). It has been speculated that the positive correlation found with mtDNA to IBD could instead be a signature of spatially discrete evolutionary lineages rather than a restriction of gene flow (Teske et al. 2018). While limitations exist for any single gene analyses, the high mutation rate,

maternal inheritance and small effective size make mtDNA more likely to track divergence than a single nuclear gene. The absence of cross-over in mitochondrial DNA provides a more straightforward analysis of genetic relatedness since inter- and intra-variability within metapopulation is reduced. Despite the ambiguity of the efficacy of mitochondrial sequences to assess population connectivity at a small-scale, the large database and ease in amplification make it a valuable tool in the genetic toolbox. Significant levels of divergence using mtDNA have shown it can be used at the population scale depending on the marker selected.

Mitochondrial DNA Analysis

To date the following mitochondrial markers have been used in genetic studies of *N. viridescens*: Cytochrome B (Johnson, 2001; May, 2011; Whitmore et al., 2013), the D-loop and flanking sequences (Whitmore et al. 2013), ND2 gene and flanking tRNA-met (Lawson and Kilpatrick, 2014). Bare (2018) used the complete mitochondrial sequence to investigate the rare black-spotted newt's population structure. For this study I used a sequence which included the D-loop sequence, tRNA-pro, tRNA-phe and part of the 12SRNA. There was an overlap of 178 bps between the sequence in this study and the D-loop sequence used by Whitmore et al. (2013). (See Table 8 for a summary of study locations, genetic markers and subspecies)

N. viridescens' studies using mtDNA have demonstrated its usefulness in detecting differentiation between populations. Whitmore et al. (2013) identified sequence differentiation between two lineages of central newts dating from the early Pleistocene showing a ~4% divergence across Cytochrome b and D-loop sequences. They concluded

that the detection of persistent genetic isolation indicated a lack of continuity between the regions. Whether this prolonged, continued separation is a result of natural habitat discontinuity or can be attributed to more recent anthropogenic habitat alteration has yet to be investigated. They speculatively concluded that the genetic patterns observed most likely could be caused by reduced dispersal capabilities. Lawson and Kilpatrick (2014) re-evaluated the findings of an allozyme study of *N. viridescens* subspecies conducted by Gabor and Nice (2004) using the mitochondrial sequences, ND2 and flanking tRNA-met genes. They confirmed the findings that haplotypes are grouped by geographic region and not taxonomic designation. They suggested that the origination of these genetically distinct groups dated back to the Last Glacial Maximum (Lawson and Kilpatrick, 2014). Using mtDNA (Cyt-b), May (2011) found evidence that eastern and western populations of the striped-newt to be distinct with no recent indication of genetic exchange. Using a full mitochondrial sequence Bare (2018) found that sequences diverged by just 2% in populations of the black-spotted newt. These findings of limited dispersal ability inferred from distinct genetic clusters using mtDNA analysis may instead be attributed to existence of lineages previously isolated in glacial refugia and should be considered speculative until supported with other genetic markers.

Microsatellite Analysis

To obtain a more detailed view of population structure a multi-locus nuclear approach should be used in conjunction with mtDNA analysis. Teske et al. (2018) found that microsatellite datasets were more likely to detect IBD. Microsatellites also known as "Short Sequence Repeats" (SSRs) are DNA sequences consisting of tandem repeats of

short nucleotide motifs (1 to 6 or more base pairs). Because of their high mutation rates with high levels of polymorphism within the same population they are better suited to detect IBD (Benson 1999; Goldstein and Schlotterer 1999). Two sets of nuclear microsatellite markers have been identified and used in population studies of *N. viridescens*. Croshaw and Glenn (2003) isolated seven microsatellite DNA loci from two populations of newts in South Carolina. They proposed that the high heterozygosity of five of the loci made them potentially useful tools for future population studies. Whitmore et al. (2013) used three of these markers in conjunction with mitochondrial sequences, including a portion of the D-loop, to investigate genetic patterns in *N. v. louisianenesis* in Iowa. For use in a study of the striped newt May (2011) developed and utilized 9 microsatellite markers.

Table 8 Summary of *Notophthalmus viridescens* genetic studies

Study	Geographic Region	Sub-species	MtDNA Marker	Nuclear DNA
Johnson 2001	Northern Florida	Striped Newt	Cytochrome b	
Croshaw and Glenn 2003	Aiken County South Carolina	Red-spotted newt Striped newt Central newt		Developed seven microsatellites
May et al. 2011	Northern Florida and Georgia	Striped Newt	Cytochrome b	Developed nine microsatellites
Whitmore et al. 2013	North-eastern Iowa	Central Newt	D-loop and tRNA; Cytochrome b	Microsatellites (Croshaw and Glenn, 2003)
Lawson and Kilpatrick 2014	Locations throughout South Carolina	Red-spotted newt Striped newt Central newt	ND2 and tRNA-Met	
Bare 2018	Texas and Mexico	Black-spotted Newt	full mitochondrial sequence	

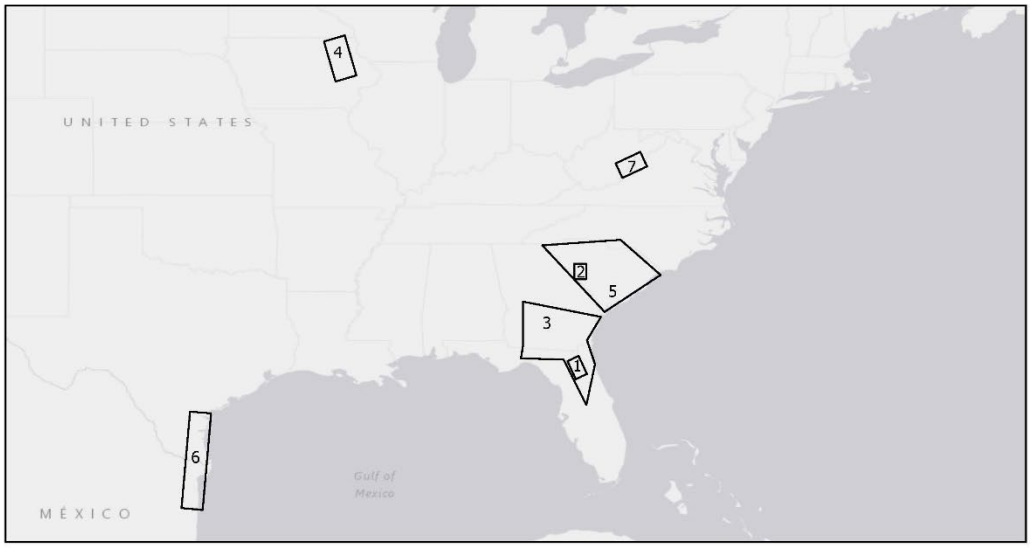

Figure 10 Geographic areas of genetic studies of N. viridescens.

Areas are approximations and do not indicate the number of surveyed sites within each area. For genetic markers used and sub-species evaluated refer to Table 8. 1) Johnson (2001) 2) Croshaw and Glenn (2003) 3) May 2011 4) Whitmore et al. (2013) 5) Lawson and Kilpatrick (2014) 6) Bare (2018) 7) Shaffer (2021).

Croshaw and Glenn Microsatellites

For this study I initially attempted to use the microsatellites published by Croshaw and Glenn (2003). I was unable to get reproducible results for any of the microsatellites despite extensive attempts to optimize the PCR. Inconsistent results were initially credited to the use of skin swabs versus tissue. However, the use of two tissue samples as controls did not improve the reliability of the outcome. The chromatographs of those samples that I was able to successfully amplify were not consistent with expected microsatellite results (Figure 11). Multiple distinct peaks were observed beyond the typical stutter pattern seen in microsatellites leading to the conclusion that I was looking at a tetrapoid organism. Whitmore et al. (2013) reported that only three of these seven loci showed reliable scoring patterns (*Nvi*2, *Nvi*7, and *Nvi*11). Of these three they found *Nvi*2 to show the lowest level of polymorphism.

Methods

For a complete description of the study sites and the DNA extraction method refer to Chapter Two. The DNA was amplified using fluorescently labeled microsatellite primers published by Croshaw and Glenn (2003). The PCR was conducted in a total volume of 20 µl containing 2.0 µl of DNA template, 2 µl 10× buffer with $MgCl_2$, 1 µl primer 1.0 F (10 mM), 1.0 µl primer R (10 mM), 2.0 µl dNTPs (2 mM each), 2.0 µl BSA (0.01%), 0.1 µl Taq Gold polymerase (5 U/µl) and 7.9 µl DEPC water. PCR conditions consisted of an initial cycle at 95°C for 11min, 40 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products that yielded clear band PCR products were discriminated using an ABI-Prism Genetic Analyzer.

Allele sizes were determined relative to the size standard and scored using the GENEMAPPERTM 4.1 software.

Results

Of the microsatellites I tested only Nvi2 yielded clear reproducible chromatograms consistently. Chromatograms for microsatellite Nvi2 (Figure 11) showed multiple alleles indicating the possibility of a tetraploid genome; chromatograms A, B, D, and E appear to be polyploid; A and C are tetraploid, and B and E are triploid. C was included as an example of one of the many inconclusive chromatographs presumably attributed to weak PCR products. The chromatograms showing triploidy (B and E) are from individuals from the same pond located in the Massanutten mountain range. Duplicate chromatograms of repeated PCRs for DNA samples confirmed these genotypes, as seen in Figure 12 which shows the repeated PCR of DNA extracted from a skin swab.

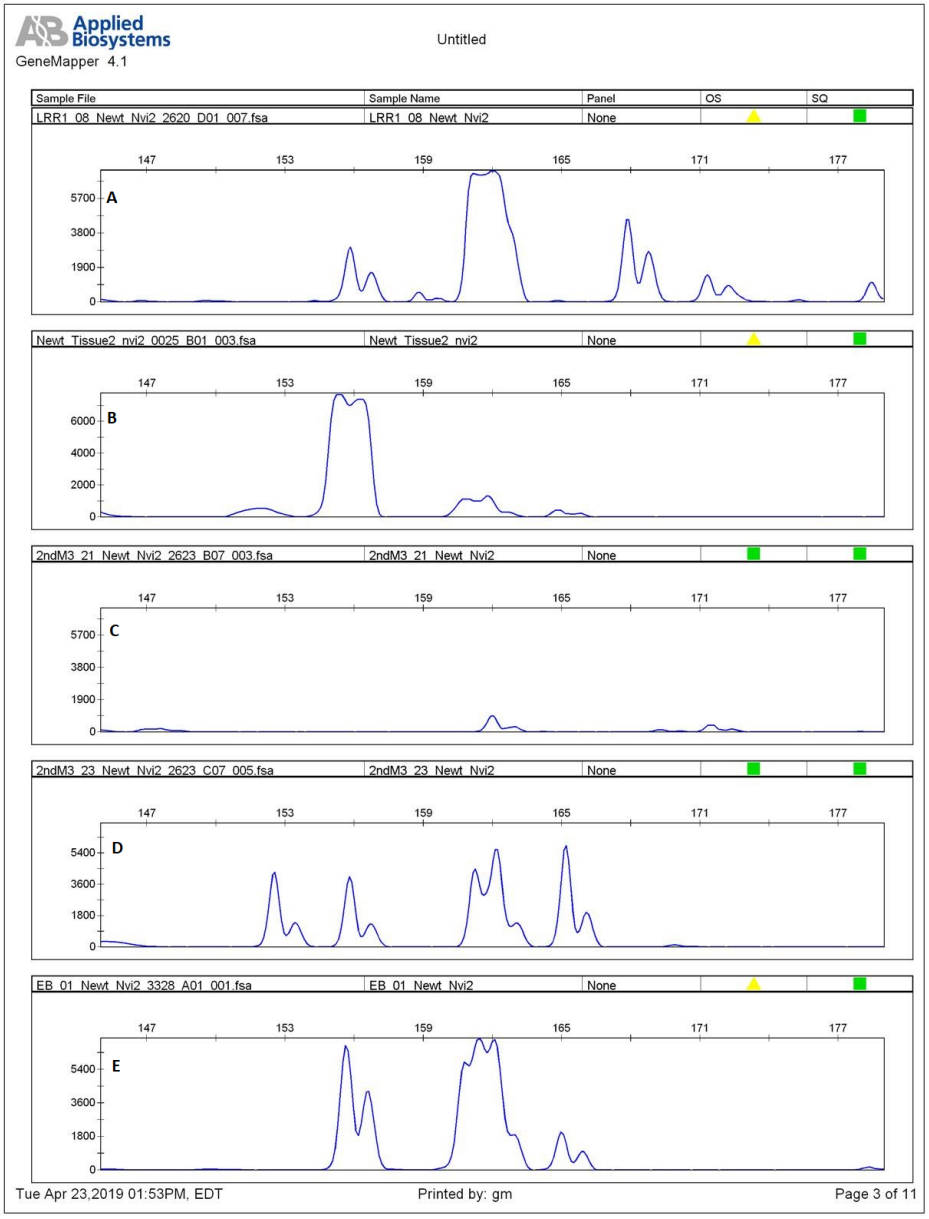

Figure 11 Chromatograms of Croshaw and Glenn microsatellite nvi2

The genotype plots of Croshw and Glenn (2003) microsatellite Nvi2 generated using The GeneMapper® Software Version 4.1 show multiple alleles at single locus.

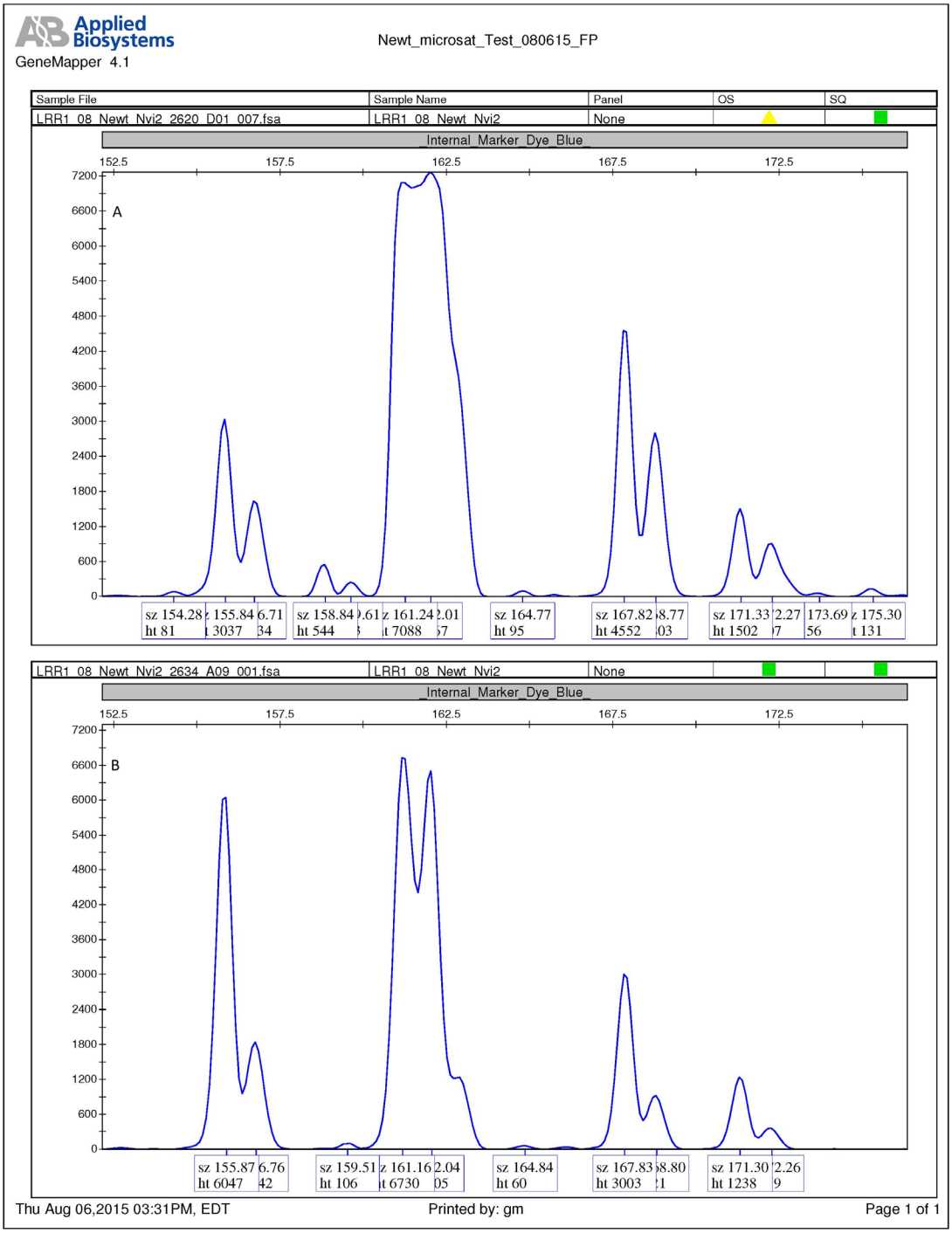

Figure 12 Chromatogram for repeated PCR of LRRI_08 (nvi2)

Development of New Microsatellite Markers

Methods

Sequencing

After unresolved issues with the Croshaw and Glenn microsatellites, I used next generation sequencing (NGS) and Ion Torrent technology to isolate microsatellite sequences from the tissue samples of two red-spotted newts. DNA was extracted from tissues using the Qiagen DNeasy Blood and Tissue Kit. Except for an increase in protease digestion time to 1 hour, all standard protocols from the manufacturer were followed. The genomic DNA extracted was sequenced with the Ion S5 NGS instrument (Applied Biosystems Inc.). Libraries were prepared using the IonXpressPlus gDNA Fragment Library preparation kit which included the following steps: Fragmentation of DNA and ligation of adaptors to DNA fragments, size selection with E-gel, amplification and purification of the final products and quatification of the final products using Qubit. Libraries were analyzed for size, quality and concentration using Bioanalyzer. The samples were run on Chef for amplifcation and then sequenced using the S5.

Microsatellite identification and primer design

Over twenty three millions reads were generated using the S5. Because the small size of the reads, less than 310 bps (appendix figure 16), would have made the likelihood of isolating microsatellites prohibitive, contigs were assembled using SPAdes. From the assembled contigs, 63 (Figure 17) were analyzed for potential microsatellite loci using the program *msatcommander* (Faircloth 2008). I identfied 63 potential microsatellite

locus primer pairs. From these possibilites 10 were selected for testing based on the fragment length and the number of motif repeats.

Primer test

A total of 10 primer pairs was selected as potential microsatellite markers (Table 9). PCRs for each pair were conducted in a total volume of 20 µl containing 2 µl of DNA template, 2 µl 10× buffer with $MgCl_2$, 1 µl primer 1 (10 mM), 1 µl primer 2 (10 mM), 2µl dNTPs (2 mM each), 2µl BSA (0.01%), 0.1 µl Taq polymerase (5 U/µl) and 9.9 µl DEPC water. PCR conditions consisted of an initial cycle at 95°C for 11min, 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products that yielded a clear band on 2% agarose gel by electrophoresis were purified and analyzed using an ABI DNA analyzer (Applied Biosystems, Foster City, USA).

Results

Of the ten microsatellite primer pairs tested, PCR product from tissues was verified for four (2110, 9329, 9917, 1726) through electrophoresis on 2% agarose gel (Figure 13). However when PCRs using DNA extracted from skin swabs were performed for these successful microsatellites only microsatellite 9917 resulted in strong product and only for some of the samples. Only weak product resulted for microsatellite 2110. (Figure 14)

Chromatographs of these successfully amplified DNA samples also showed multiple peaks (Figure 15) confirming the inference of polyploidy made from the Croshaw and Glenn microsatellite chromatographs. The sample shown in chromatogram B

(LRR1_08) was also found to show four peaks in the Croshaw and Glenn microsatellite chromatogram, A in Figure 11 and 12.

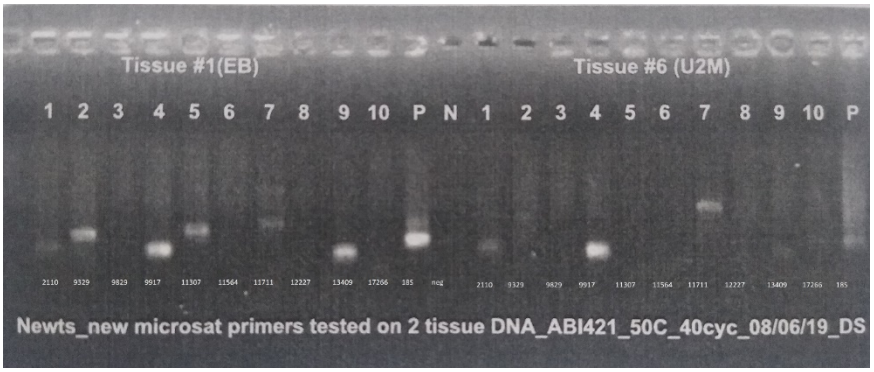

Figure 13 Gel electrophoresis for all microsatellites using tissues

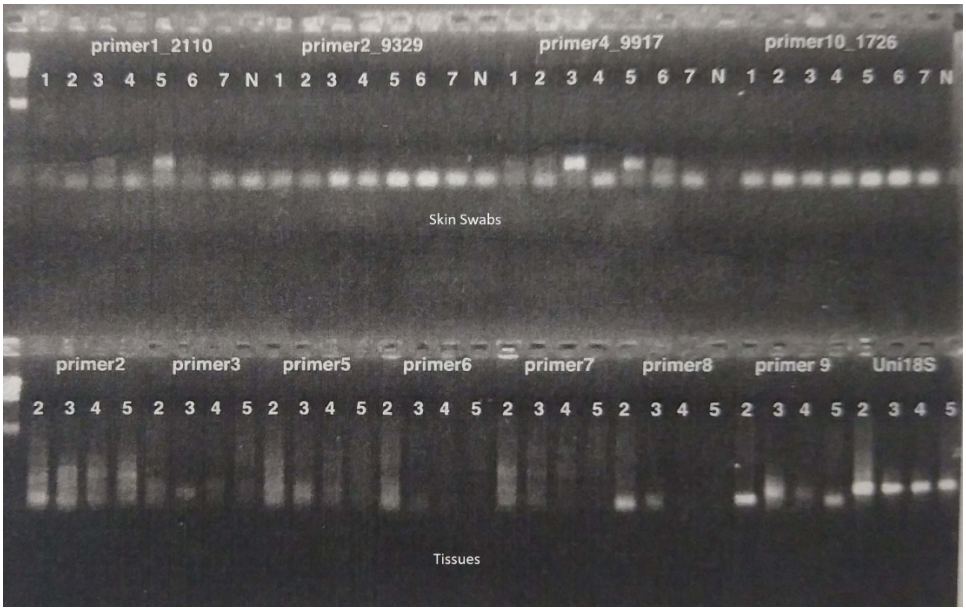

Figure 14 Gel electrophoresis comparing tissue and skin swabs for microsatellites

(Top row) Gel electrophoresis results on 2% agar using DNA extracted from skin swabs for microsatellites 2110, 9329, 9917, and 1726. These four microsatellites were successfully amplified using DNA extracted from tissue samples. (Bottom row) Gel electrophoresis for DNA extracted from remaining tissue samples for seven of the primer sets.

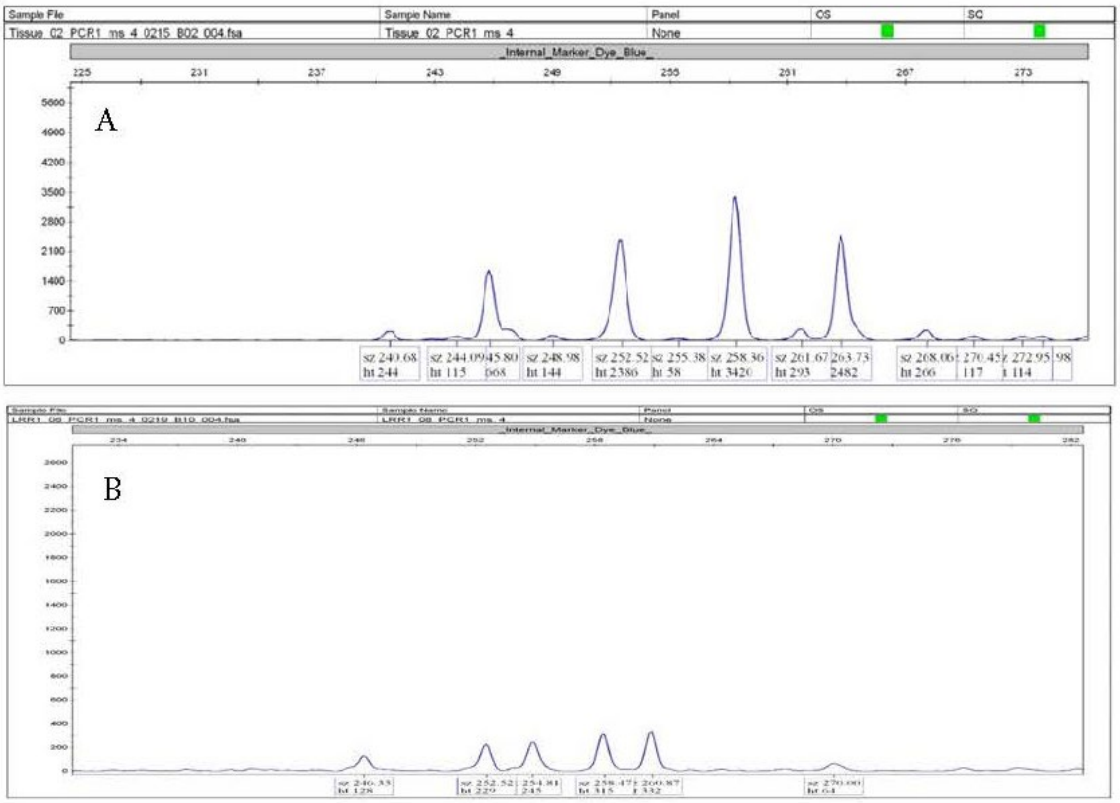

Figure 15 Chromatogram of microsatellite 9917

Chromatogram A was from DNA extracted from a tissue sample and chromatogram B was run from DNA extracted from a skin swab.

Discussion

The low success rate of amplification of both sets of microsatellite markers prevented their use as part of a population study. However, preliminary results from the evaluation of these markers show evidence of polypoidy in the newts. The Croshaw and Glenn chromatograms show the presence of four alleles at a single locus for multiple samples (Figure 11). Chromatograms from our unproven microsatellites also showed multiple peaks indicating polyploidy (Figure 15). Fankhauser (1938) found that out of a hundred *N. viridescens* larvae analyzed, four were triploid and the remaining ninety-six were diploid. Heat induced triploidy in *N. viridescens* was observed by Fankhauser and Watson (1942). Among sexually reproducing animals, polyploidy is rare. In a classic discussion in 1925, H.J. Muller argued that "a most remarkable concatenation of events must obtain before a persistent tetraploid line can actually become established, and capable of surviving in a state of nature, in animals having the prevalent type of sex determination." Among the vertebrates the only known examples of polyploidy are the triploid larvae of frogs and salamanders. While tetraploidy has not been previously reported for *N. viridescens*, variable ploidy, including high levels of tetraploidy have been found in populations of *Ambystoma jeffersonianum* (Phillips 1997). Experimental induction of polyploids has been conducted on amphibians since the late 1930's. Given these findings and the large number of diploid eggs produced by amphibians, the presence of natural polyploidy is not unexpected. The lack of detection may simply be due to a lack of experimental observation. The tetraploid state found in the populations surveyed for this study may be unique, however given the very low numbers of *N.*

viridescens individuals that have been analyzed to date overall this remains uncertain. Additionally, given the prevalence of tetraploid results I found, tetraploidy would not be a rarity in this population. Future research into the ploidy level of *N. viridescens* should be conducted either through further microsatellite assessment or cytogenetic analysis throughout its range.

The issues encountered with the amplification of microsatellite markers was most likely linked to the use of skin swabs. The successful use of microsatellite markers in other population studies of *N. viridescens* were conducted using DNA extracted from tissue samples. Amplification using six available tissue samples resulted in strong product for several of my potential microsatellites (Figure 13). However when DNA from skin swabs were amplified the results were inconsistent, frequently showing weak or no product. (Figure 14). According to Pidancier et al. (2003) microsatellite genotyping using buccal swabs for amphibians was unsuccessful when the DNA was kept at room temperature but successful if the DNA was fresh or frozen. Prunier et al. 2013 found that skin swabs of *Ichthyosaura alpestris* could successfully be used for microsatellite analysis but did not assess the degradation potentially associated with frequently thawed DNA. Although the DNA I used was stored frozen, the thawing and refreezing during usage may have degraded the DNA. Low quality DNA has been shown to increase genotyping errors with microsatellite markers decreasing the reliability of genetic analysis (Taberlet et al. 1999). To ensure valid results a multiple tube approach has been suggested (Goossens et al. 1998). The inability to reliably duplicate the microsatellite reactions using our skin swabs led to the decision to switch to a mtDNA marker for our

population study of the red-spotted newt. Pidancier et al. (2003) found that genotyping of mtDNA stored at room temperature was more likely to be successful than microsatellite makers; amplification and sequencing of mtDNA cytochrome b gene was successful for DNA stored at room temperature for *Triturus cristatus, Rana temporaria*, *Salamandra atra* and unsuccessful for *Salamandra salmandra*, *Rana esculenta*, and *Salamandra salamandra* (Pidancier et al. 2003). Given the prior issues encountered with the Croshaw and Glenn microsatellite markers and the fact that these species may be polyploid, our microsatellites were not fully evaluated. The possibility that these microsatellite markers may prove useful in future studies warrants further effort.

Recommendations for Future Research

Since genetic population studies have focused on the rarer subspecies of *N. viridescens* at the edge of the range, recommendations for further research would be to investigate the red-spotted newt populations more centrally located. To facilitate the comparison of results across studies, the use of common and multiple mtDNA markers in *N. viridescens*' research should be considered. Additionally, mtDNA studies of *N. viridescens*, should be re-evaluated with a multi-locus approach. The structure inferred from a single locus may not provide the detailed information a multi-locus approach can. Goudarzi et al. (2019) used Restriction Associated DNA sequencing (RADseq) to analyze populations of *Neurergus kaiseri* previously assessed using the mitochondrial D-loop. The existence of two clades indicated by mtDNA was corroborated using RADseq, but the finer detail allowed them to better identify barriers to gene flow. The higher resolution and truer history of populations made possible with multiple loci can augment

the knowledge gained from mtDNA analysis alone. Geographic expansion of study areas and a more comprehensive genetic analysis using a multi-locus approach and comparable genetic markers are necessary to make future informed conservation decisions based on an accurate assessment of the population genetic structure and the contributing processes shaping it.

APPENDIX

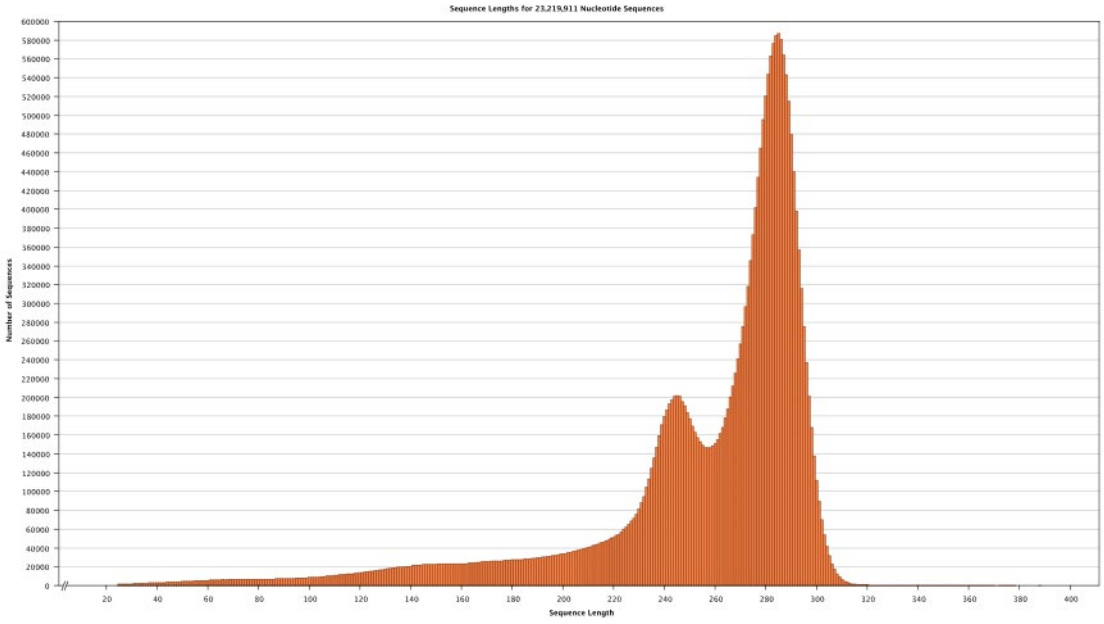

Figure 16 Sequence Lengths for Reads from Ion Torrent Sequencing

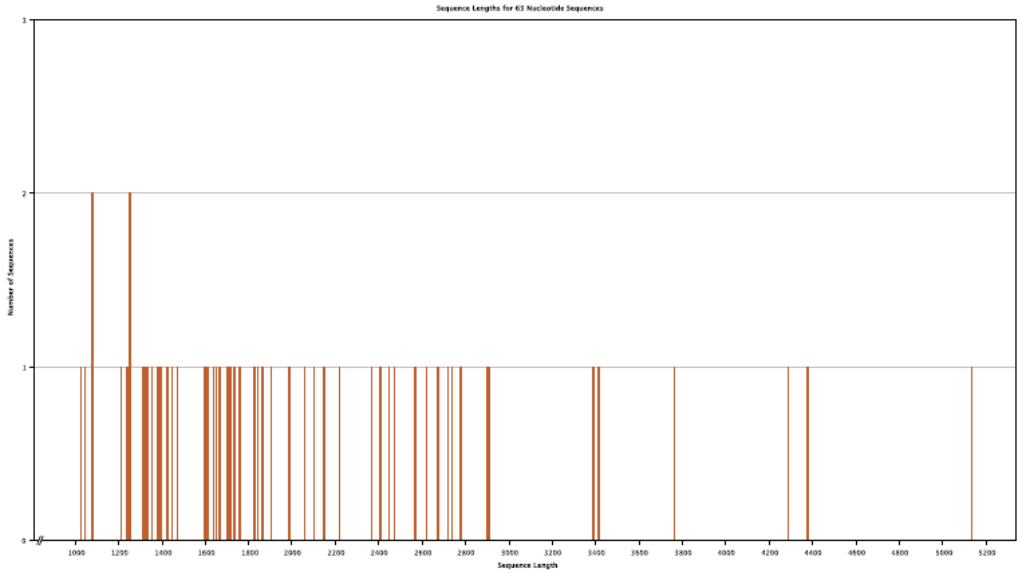

Figure 17 Sequence lengths of SPAdes assembled contigs

Table 9 Primers identified for potential microsatellite markers identified in this study for *N. v. viridescens*

Locus	Direction	Primer sequence	T_m	Size	Repeat motif
Newts_2110	F	TAACAAAGAACTCCAGGACC	49.7° C	285	$(AT)_9$
Newts_2110	R	ACATTTGGTGAAGATTCGTC	47.7° C		$(AT)_9$
Newts_9329	F	CAAGAACTCACTCTAGCAGG	51.8° C	285	$(AGAT)_9$
Newts_9329	R	CTAATAGGGAGTTTGGGACC	51.8° C		$(AGAT)_9$
Newts_9829	F	TTCACTAAGGCCTACTCTTC	49.7° C	258	$(AT)_9$
Newts_9829	R	TTTGCCTCTGAAACACAAAG	47.7° C		$(AT)_9$
Newts_9917	F	AGAATCCGACTGTTGTAGAG	49.7° C	270	$(AGG)_8$
Newts_9917	R	CTTCAGATACCTCCAGAGAC	51.8° C		$(AGG)_8$
Newts_11307	F	TGGATTGGGATACTTACATG	47.7° C	284	$(AT)_{10}$
Newts_11307	R	TGAGGTAAGAACATGAGGTC	49.7° C		$(AT)_{10}$
Newts_11564	F	TATCCATCCATTCACTAGCC	49.7° C	233	$(AC)_{15}$
Newts_11564	R	ACTCTGTCACTTAGTACGTG	49.7° C		$(AC)_{15}$
Newts_11711	F	TTCTTCTTACAATCATGCCC	47.7° C	275	$(AGAT)_{14}$
Newts_11711	R	ACTGCAACACACTTTCATAG	47.7° C		$(AGAT)_{14}$
Newts_12227	F	ACCTAAGAAGATACTGGTGC	49.7° C	223	$(ACC)_8$
Newts_12227	R	TCTGATGGATCTGGTGTAAC	49.7° C		$(ACC)_8$
Newts_13409	F	GTGTCGATATTGAGTGGTTG	49.7° C	296	$(AT)_{10}$
Newts_13409	R	AACGTAAATCCTCCAGTGTG	49.7° C		(AT)10
Newts_17266	F	AGTAAGCACTTCAGACCTAC	49.7° C	181	$(AC)_{18}$
Newts_17266	R	CATCGCAGGTAGAAGTCTC	51.1° C		$(AC)_{18}$

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