USING DNA FINGERPRINTING TO ASSESS THE GENETIC STRUCTURE OF THE VERNAL POOL AMPHIBIAN *Rana sylvatica*

by

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DEDICATION

Although I owe the completion of this project to the generous help of many individuals, there is one who was always at my side, through the thick and the thin, the highs and lows, the hot and the cold. Over the years we certainly have had our differences, but we always worked through them. Your presence eased the burden of many long days in the lab and although I know not where the path of life will lead me- unfortunately cannot accompany me. I could go on for pages praising your virtues, but it is time for us to part ways, know that you will be missed. Thank you, Mr. Slim®.

ABSTRACT

In this study, I used restriction fragment length polymorphism (RFLP) analysis (DNA fingerprinting) to study the genetic population structure of wood frogs, *Rana sylvatica*, collected as egg masses from vernal pools within the Massachusetts Audubon Society Lincoln Woods Wildlife Sanctuary in Leominster, MA. The average genetic relatedness of sibling individuals, non-sibling individuals from within the same pool, and individuals from pools of close (5 m), far (200 m) and distant (40 km) spatial separations was calculated. The goal was to use genetic relatedness to estimate the breeding patterns of *R. sylvatica* and use that information to make general management recommendations that could be applied to other vernal pools breeders.

I detected relative differences in genetic similarity between individuals from pools only 5 meters apart, however over a larger distance of 200 meters no significant genetic differences were present. This suggests that although wood frogs are known to be highly philopatric, they may use information other than simply proximity to their natal pool when choosing breeding sites. Factors such as species composition, water chemistry and heterogeneity of the landscape between pools may influence breeding site choice. Also, contrary to the findings of recent studies, the distance between vernal pools may not be the best indicator of the genetic similarity of the individuals they host. Pools in close proximity to one another may host genetically distinct populations, and therefore management decisions should be made on a pool-by-pool basis. Consequently, when managing populations of *R. sylvatica,* and possibly other vernal pool breeders, taking into account parameters other than simply the spatial separation of pools within an array may avoid decisions that could result in the loss of genetic diversity.

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BACKGROUND

A global concern

Over the past two decades much attention has been drawn to the apparent worldwide decline in amphibian populations. Growing amounts of anecdotal evidence caused the National Research Council to organize a workshop and subsequently form the Task Force on Declining Amphibian Populations (DAPTF) in 1990 (Wake, 1998). Although global variation appears great, preliminary reports of the DAPTF, as well as those of numerous outside researchers, suggest amphibian populations to be unstable at best, with multiple species in decline and a number of documented extinctions (Kiesecker *et al.*, 2001; Wake, 1998). Although the presence of breeding populations of amphibians can sometimes impart a special regulatory status to a habitat, it has been found that even protected habitats have experienced losses. A study of nineteen anuran species, spanning protected areas from Australia to South America, declared the observed population declines as "a serious global concern" (Berger *et al.*, 1998).

Currently one of the biggest problems in researching the causal effects of amphibian declines is dealing with confounding anthropogenic and natural factors and trying to examine them in the context of seasonal population fluctuations (Pechmann *et al.* 1991). The list of studied causes for population loss is quite varied and includes pollution (*e.g.* acid rain, pesticides), increased ultra-violet radiation, introduction of exotic competitors and predators, and habitat exploitation for development, logging and mining. Of these, habitat destruction is widely accepted as the most detrimental and a major cause of amphibian population decrease (Blaustein *et al.*, 1994; Smith *et al.*, 2000;

Wake, 1991). It seems that effective habitat conservation and management is a good first step in stabilizing amphibian populations.

There is, however, a great deal of research that remains to be done. The majority of population stability studies conducted has been rather short, lasting fewer than 10 years, with some fewer than 5 years. It has been suggested that these shorter studies, even when encompassing a complete turnover of a population, will underestimate the variability present simply because of large but infrequent natural fluctuations in population size (Blaustein *et al.*, 1994). Blaustein also notes that only after long-term studies are done will there be reliable baselines for the study of amphibian population dynamics.

Amphibians are a crucial component of the diverse ecosystems they inhabit, serving a number of roles. Most amphibians live anywhere from a few months to several years in a larval stage prior to becoming pre-reproductive juveniles and reproductive adults. This is important because over their life history they can strongly contribute to multiple levels within a trophic structure. As aquatic larvae they can serve as important herbivores as well as a valuable source of prey (Blaustein *et al.*, 1994; Dickman, 1968). As adults they also serve as prey, but in addition have been found to be top carnivores, consuming insects and other invertebrates. It has also been suggested that they can comprise the majority of vertebrate biomass in some systems (Blaustein *et al.*, 1994; Petranka *et al.*, 1993; Wake, 1991). Consequently, it is thought that a loss of amphibian populations could have a detrimental effect on many of the species that coexist with them.

In addition, many believe amphibians serve as indicator species, or bioindicators, that can give insight to the general state of the environments they inhabit (Wake, 1991). Their skin is highly permeable, being the main organ of osmoregulation, and can readily uptake any contaminants from their surroundings (Ralph, 1978). This is one of the reasons many amphibians are especially susceptible to even small changes in their local habitats or climates (Demaynadier *et al.*, 1998). Due to their highly sensitive nature, amphibians would likely be one of the first groups of organisms to exhibit a negative response to environmental stresses (pollution, global warming, etc.); which leads one to understand why their apparent global decline has caused such alarm in the scientific community as well as the popular media.

Management of fragmented populations and island biogeography

One of the challenges facing conservation biologists is to conduct studies that will lead to the creation of management plans based on the specific needs of species and the unique habitats they occupy. A number of amphibians such as salamanders and frogs, including the study organism for this paper, the wood frog *Rana sylvatica*, breed in isolated habitats, such as vernal pools. While vernal pools naturally occur throughout areas of continuous forest, the breeding populations that they host are physically separated from one another. It is accepted that the structure of such fragmented populations is predicted by the theory of island biogeography.

Island biogeography theory states that the equilibrium species number on an island should increase with island area and decrease with island distance to the source population (MacArthur & Wilson, 1963, 1967). Island biogeography theory was

developed through the study of oceanic islands but is applicable to any habitat "islands", meaning patches of suitable habitat separated from one another by uninhabitable areas (Wiggins *et al.*, 1998). "Island" habitats can be extremely diverse with examples ranging from forest fragments, individual host flora and, as will be seen in this study, vernal pools. Inherent within this theory is the concept that there is some degree of species turnover between habitat patches; meaning species may experience periodic local extinctions with the possibility of recolonization. Also, when a species becomes extirpated from a particular fragment, its recolonization by individuals from neighboring fragments is dictated by island biogeography theory. Hypothetically, the larger a recently extirpated patch is and the closer it is to occupied neighbors, the sooner it will be recolonized through immigration. However, there are many other factors that affect migration between islands, which may be species-, habitat- or case-specific.

In dealing with species in fragmented habitats, assessing the degree of interpopulation migration is essential to effective management. Migration between habitat fragments is a defining characteristic of these populations and the degree to which it exists can affect multiple population parameters, including gene flow, genetic drift, inbreeding depression, adaptive capacity and overall genetic diversity. When populations persist in reproductive isolation from one another, genetic distinctions may begin to form. In the extreme case, this will manifest as speciation between two previously conspecific groups. There are numerous historical records of speciation events occurring across archipelagos, such as the Galapagos and Hawaiian islands, where barriers to migration are great and colonization rarely occurs (Wilson, 1992).

On a smaller spatial scale, reproductive isolation can lower the overall genetic variability of a population. Due to habitat destruction and degradation, organisms that once enjoyed a continuous environment with equal access to all breeding partners may now be fragmented into a number of smaller groups over the same landscape. Assuming less than ideal corridor structure and some degree of impassable barriers, migration will not be optimal and a number of smaller interbreeding groups will result. Isolated populations such as these can experience a drop in genetic diversity as a result of genetic drift, reduced gene flow, differential selective pressures and smaller overall breeding numbers (Gibbs, 1998; Johnson *et al.* 2000). Individuals within these populations may also experience reduced heterozygosity through a rise in inbreeding (Buza, *et al.* 2000).

The reduction of genetic variability that isolated populations may experience, at the level of group or individual, can result in the loss of rare alleles and reduced adaptive ability (Bridges & Semlitsch, 2001; Wilson, 1988). When faced with environmental or anthropogenic perturbation, the response of a population will be determined by its phenotypic capacity to deal with that stress. If the stress is great enough, members of the population will die, potentially resulting in extinction. Assuming a number of individuals survive for reasons other than sheer chance, the resulting population structure will be based on the genetic ability they possessed to adapt to and deal with change. This hypothesis is based directly on Darwin's theories of natural selection and the underlying ability of organisms to pass favorable phenotypes on to offspring, assuming said traits are genetically heritable (Darwin, 1859).

Mortality studies have been conducted on amphibian populations with respect to agricultural chemicals (Bridges & Semlitsch, 2001), fungal parasites (Berger *et al.*, 1998)

and other factors known to exist in the field. Variable mortality rates among groups from different localities or families can be explained by genetic variability. Individuals within these populations possess differential genetic capacity to respond to environmental stresses. When dealing with isolated populations, it would be conservationally prudent to assess overall genetic variability across a metapopulation when making management decisions. A metapopulation is a group of local populations connected to one another by the dispersal of organisms between them. The introduction of an invasive factor, such as a pollutant, disease or exotic competitor/predator/parasite, into an amphibian ecosystem could have devastating effects on individuals lacking the phenotypic capability to deal with such changes.

Consider a metapopulation with a great deal of inter-patch breeding which results in successful reproduction overall. Over time the result of this high degree of interbreeding will be a near panmictic genetic structure among the population fragments. Over time there will be fewer individuals in the population likely to possess a rare or unique allele conferring some adaptive ability in defense of a catastrophic event. An event capable of removing some individuals in the population could likely affect them all since everyone should be genetically similar The other extreme is the situation where individuals of a population are extremely philopatric and rarely, if ever, crossbreed to neighboring groups. While this situation was cited earlier as a mechanism of speciation, over shorter temporal scales the more likely outcome is the formation of a number of genetically distinct subpopulations. The arrival of a similar catastrophic event could have varying effects on different subpopulations, possibly wiping out some and leaving others seemingly unaffected. Rare or unique alleles would have a better chance to propagate

and persist within a fragment due to smaller breeding groups, however these once rare alleles would only be present in a relatively small number of individuals. In the event that catastrophe eradicates individuals from some fragments, the possibility of recolonization from surviving patches exists.

In a study of southern leopard frogs, *Rana sphenocephala,* Bridges and Semlitsch (2001) wrote, "The amount of genetic variation and its long-term maintenance (within a population) is critical to understanding adaptation for changing environmental conditions". Genetic variation is maintained through gene flow, which can be viewed as having two main components: (1) dispersal of individuals away from home sites; and (2) successful reproduction following dispersal (Berven & Grudzien, 1990). This concept is useful to fragmented habitat management if the relative genetic diversity of a population can be determined. If gene flow between patches is high, then all subpopulations share a broad range of genes. However a catastrophic event resulting in the death some individuals could likely affect everyone. On the other hand, when numerous genetic subpopulations are present, single fragments can be individually more susceptible to local extinction, but the overall population is less likely to suffer catastrophic extinction (Shaffer *et al.*, 2000). When drawing management plans for a species that exhibits high degrees of gene flow between geographically separate groups, theoretically the loss of a single habitat fragment should not dramatically alter the overall genetic variability of the population. Conversely, in the case of a highly philopatric species it would make sense to preserve each habitat fragment as supporting its own genetic entity. The loss of any one group could result in a reduction of genes and drop in genetic variability.

As was previously mentioned, the degree of movement of breeding individuals between geographically separate groups within a species is paramount in determining its genetic diversity. Recently, many studies have focused on assessing the genetic structure of fragmented populations (Buza, *et.al*., 2000; Gibbs, 1998; Johnson *et al.*, 2000; Newman & Squire, 2001; Shaffer *et al.*, 2000) for insight into migratory tendencies, with the goal of acquiring information to make informed management decisions.

Vernal pools and their management implications

In New England, vernal pools are defined as shallow, fishless bodies of water that fill to their maximum extent during winter and spring rains, and in many cases become completely dry by mid-summer. Vernal pools are a unique habitat that Massachusetts certifies and recognizes with a special regulatory status. The Massachusetts Natural Heritage and Endangered Species program can choose to certify vernal pools if they 1) display a number of physical characteristics, and 2) serve as habitat and breeding grounds for fairy shrimp, mole salamanders or other obligate breeders (MAS web site). Among these obligate breeders is the wood frog *Rana sylvatica*. The marbled salamander *Ambystoma opacum*, blue-spotted salamander *Ambystoma laterale*, and Jefferson salamander *Ambystoma jeffersonianum,* are state-listed rare species which, along with the wood frog, breed in and inhabit the vernal pools as larvae before metamorphosing and moving to upland areas as adults. In addition, invertebrate species, such as the fairy shrimp *Eubranchipus vernalis*, will complete their entire life cycles in a single season before the pools dry (Witham*, et al.*, 1998).

Due to their ephemeral nature, certification and management of vernal pools can be difficult. After the pools have dried, they can appear as no more than woodland depressions scattered with leaf litter. Although a certified pool must show some degree of regularity, it is possible for pools not to fill following a dry winter and spring. While this may cause a pool to appear to have gone out of existence, it is actually a defining characteristic of their existence.

The purpose of this thesis is to assess the genetic structure of a population of wood frogs, *Rana sylvatica,* from multiple vernal pools within an array. The degree of genetic relatedness among our experimental groups will provide insight into the dispersal tendencies and interbreeding habits of the wood frog. Our findings should allow us to make generalizations about genetic diversity between spatially separate wood frog groups. This study could assist conservation biologists making management decisions for the wood frog, and possibly other vernal pool amphibians, by providing information that will allow them to maximize the overall genetic variability of their target populations.

INTRODUCTION

Amphibian populations worldwide appear to be in jeopardy (Blaustein *et al.*, 1994; Kiesecker *et al.*, 2001; Pechmann *et al.*, 1991; Wake, 1998). Amphibians are regarded as valuable members of the environments they inhabit for a number of reasons, and there is a need for research that will contribute to the development of effective management planning for declining species. They can be key players at multiple levels within trophic structures, (Blaustein *et al.*, 1994; Dickman, 1968, Petranka *et al.*, 1993) in addition to acting as bioindicators of various environmental parameters (Wake, 1991).

A number of factors have been suggested as contributing to the loss in amphibian populations including pollution, increased UV radiation and introduction of invasive exotic species. But at this time, habitat destruction is regarded by many as the most immediate danger (Blaustein *et al.*, 1994; Smith *et al.*, 2000; Wake, 1991). Consequently, effective habitat conservation appears to be a critical first step in stabilizing amphibian populations. For many amphibian species, habitats for breeding and larval development differ greatly from those used by adults. The ephemeral wetlands known as vernal pools exemplify these specialized breeding sites.

Vernal pools are defined as temporary bodies of water that fill during winter and spring rains, and usually become completely dry by mid-summer. They are a unique habitat that Massachusetts certifies and recognizes with a special regulatory status (MAS web site). In addition to several physical characteristics, to be certified, pools must show evidence of wildlife use, which may include certain obligate breeding species, among which is the wood frog, *Rana sylvatica*. Vernal pool arrays fit MacArthur and Wilson's (1963 & 1967) definition of fragmented habitats and should be managed as such.

The wood frog, *R. sylvatica*, is distributed along eastern North America from the Appalachian Mountains to the Arctic Circle (Berven, 1990). They are obligate vernal pool breeders who exhibit extremely synchronous and precise oviposition (Berven, 1981; Wright & Guttman, 1995). Breeding typically takes place over a 1-5 day period, with males and females arriving simultaneously. In general the larval period is 60-120 days, but as temperatures rise and pools begin to dry, metamorphosis can accelerate. Males and females usually breed one to two years following metamorphosis (Berven, 1990), however under certain conditions females have been found to wait up to 4 years (Berven, 1981). Within a season, the majority of males and females breed only once with a single partner. After a breeding pair has separated, it is possible for another male to deposit sperm on the freshly laid egg mass. This secondary fertilization can result in an egg mass where some of the individuals are only half-siblings. Although there are exceptions where individuals breed for two or three years, most males and females breed only once in their lifetime (Berven, 1981).

Molecular methods, such a DNA fingerprinting (Alberte, 1994), microsatellite analysis (Newman & Squire, 2001), and DNA sequencing (Shaffer *et al.*, 2000), are gaining popularity in the field of ecology as tools to assess genetic structure among populations. With the advent of these types of studies, estimates of genetic diversity among individuals utilizing fragmented habitats, such as vernal pools, can be used when formulating management plans. Genetic diversity estimates can relate to many parameters of fragmented populations such as the degree of inter-fragment migration and breeding site fidelity (Newman & Squire, 2001). Knowledge of these varying population parameters can aid in making informed management decisions.

Previous ecological studies have suggested the distance separating populations dictates genetic variation between populations of *R. sylvatica*. A mark-recapture study by Berven and Grudzien (1990) found the average genetic neighborhood of *R. sylvatica* to exceed one kilometer, with dispersing individuals traveling an average of about 1200 meters. They concluded that there should be sufficient gene flow among pools so that all pools within a one kilometer radius of one another should show little genetic differentiation. A one kilometer radius completely encompasses all pools within our primary study site. While previous studies suggest that wood frogs disperse over relatively large ranges, they do not address which pools the frogs actually breed in. Since wood frogs, in addition to many other vernal pool breeders, are believed to be highly faithful to their natal pools and will preferentially return there to breed, molecular techniques can provide researchers with more direct estimates of interbreeding between populations. Even if a wood frog's home range contains many available breeding sites, genetic analyses can be used to detect if there is preferential choice to breed in ones natal pool.

This study was designed to see if genetic diversity between vernal pools within an array exists as a result of natal breeding pool fidelity, even when multiple suitable breeding sites are accessible. Our first goal was to develop protocols to extract suitable genomic DNA from tadpole samples as well as restrict and generate consistent DNA fingerprints from which we could identify polymorphisms between individuals. We proceeded to use multilocus restriction fragment length polymorphism analysis to assess genetic similarity of individuals within a population of *R. sylvatica.* DNA fingerprinting has been effective in resolving genetic differences between both individuals (Jeffreys, *et*

al., 1985) and populations (Alberte *et al.*, 1994). We compared the genetic relatedness of individuals from pools with varying degrees of spatial separation. Our prediction was that genetic similarity (*S*) would decrease as distance between populations compared increased. We used our estimates of genetic relatedness to infer the relative degree of interbreeding between populations.

MATERIALS AND METHODS

Study Sites

Egg masses were collected from vernal pools within the Massachusetts Aububon Society (MAS) Lincoln Woods Wildlife Sanctuary in Leominster, MA and from a single pool in a lowland area of Goss Pond in Upton, MA (Fig 1). The MAS Lincoln Woods Wildlife Sanctuary is a 68-acre sanctuary that is home to an array of six vernal pools. The pools are arranged in a 2 x 3 pattern with a large esker separating pools 1,2 & 3 from 4, 5 & 6. (Fig. 2 and 3)

Collection, care and cryopreservation of subjects

In total, eight *R. sylvatica* egg masses were collected for analysis, six from vernal pools at the Leominster site in April 2001, and two from the Upton, MA site in April 2002. Two egg masses were collected from each of pools 1, 5 & 6 from the Leominster site (called pools L1, L5 and L6 respectively). Also 2 masses were collected from the Upton pool (U1) to serve as an outgroup. Duplicate masses collected from the same pool were separated by a minimum of 1 m to increase confidence that they were from unique breeding pairs. Though not common, a second male can additionally fertilize a mass of newly deposited eggs, resulting in a mass with full- and half-siblings. Egg masses were freshly laid and no more than 72 hours old upon collection.

Egg masses were transported back to lab in vernal pool water and placed in 5-liter rectangular containers topped off with spring water. Organisms were kept at room temperature and on an ambient light:dark cycle at all times. Once the masses dissociated and tadpoles became free-swimming, individuals from each egg mass were divided

Figure 1. Map of Worcester County noting the locations of Leominster and Upton.

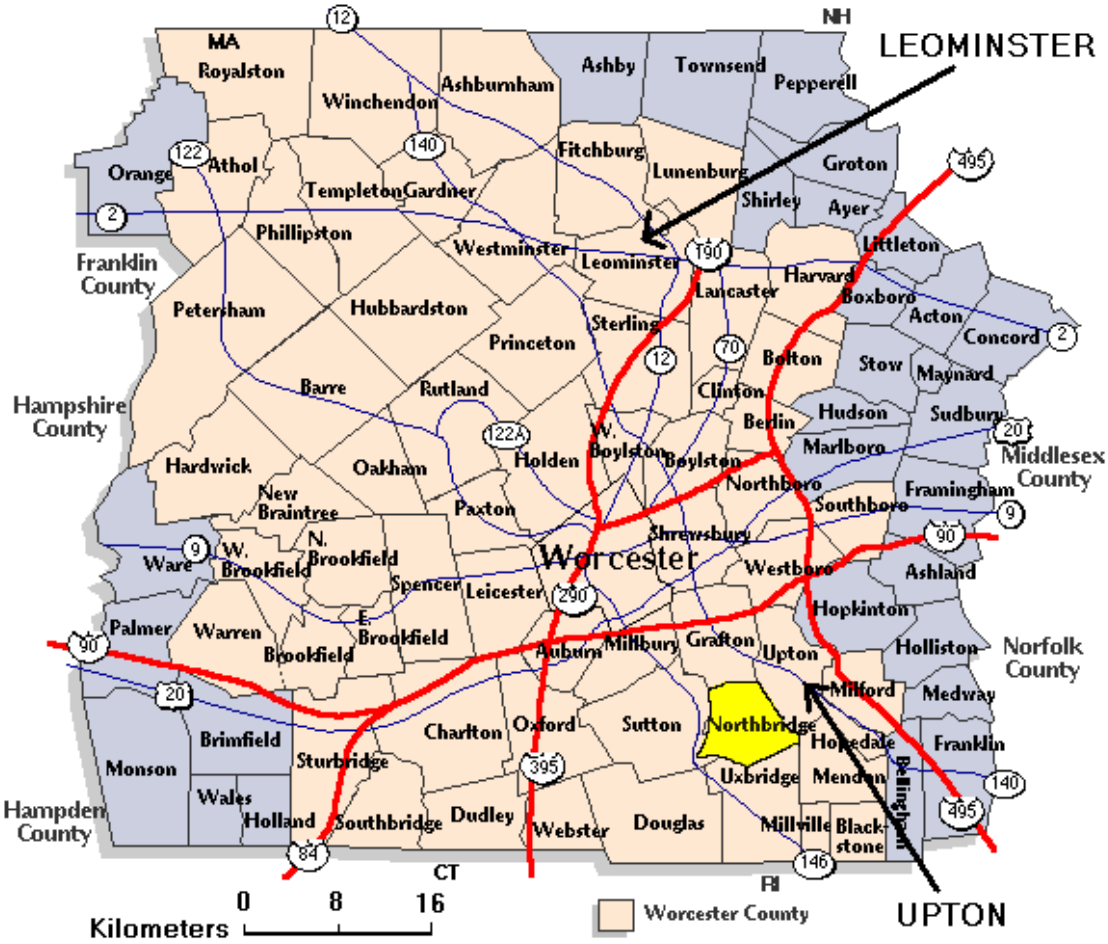


Figure 2. 1 : 25,000 topographic map of an area in Leominster, MA. The outlined region contains the Massachusetts Audubon Society Lincoln Woods Wildlife Sanctuary.

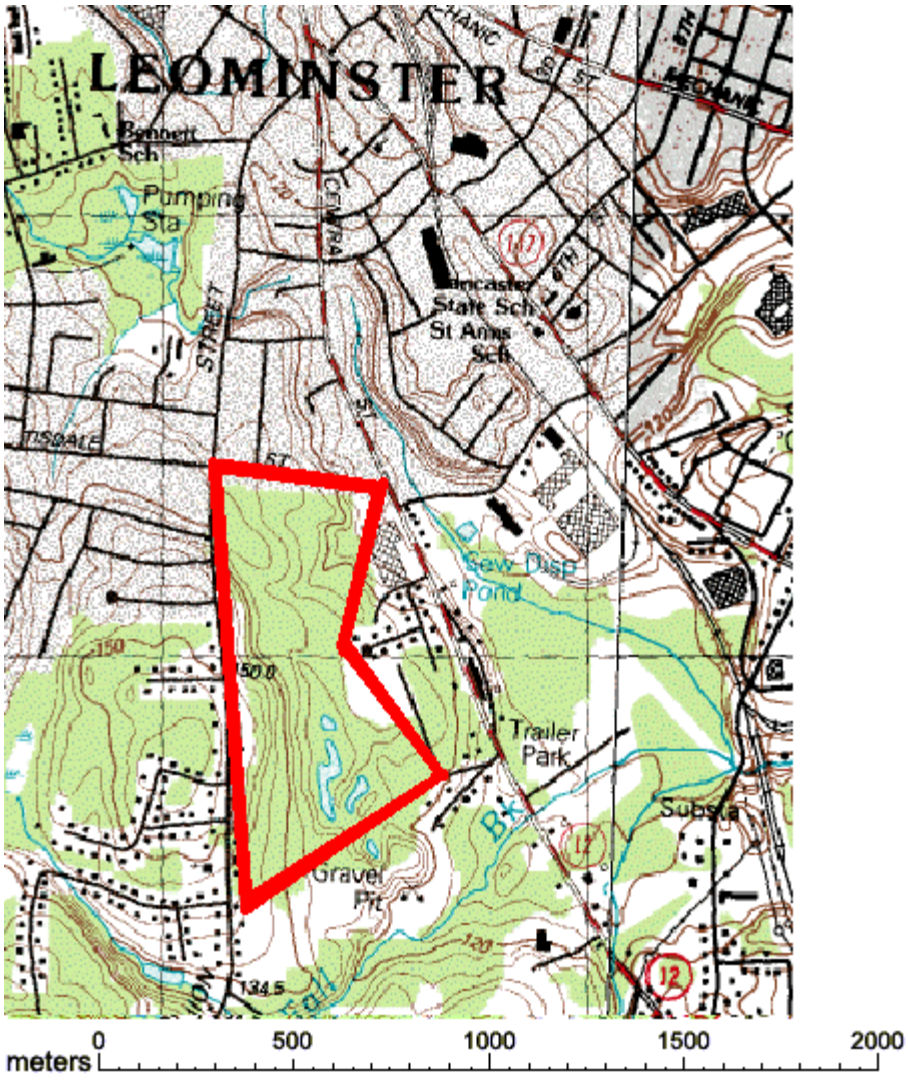
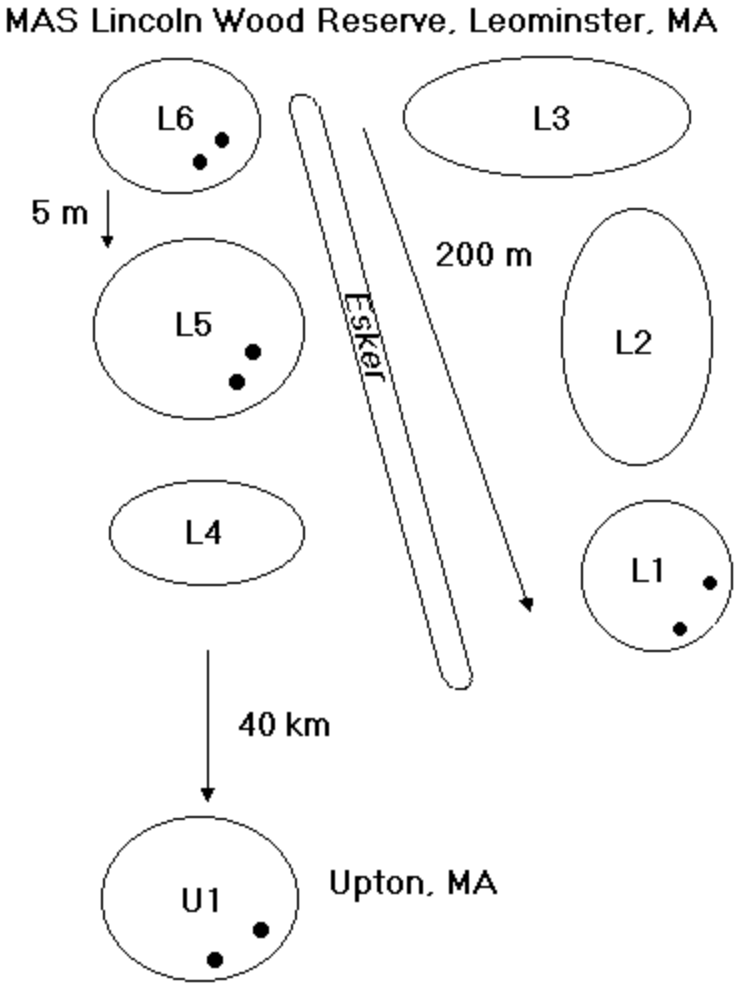


Figure 3. Schematic diagram showing the general layout of the study pools. Black circles indicate where an egg mass was collected. (Note: Figure not drawn to scale)



between 3-5 additional 5 liter containers (approximately 50-75 individuals per container). Once tadpoles reached foraging age they were fed a diet of rabbit pellets (Eight in One®) *ad libitum*. Containers received half-water changes with spring water every other day or as needed. Deceased organisms were removed immediately and feces and accumulated food matter were siphoned out as needed. Once tadpoles had grown a rather large tail, individuals were cryopreserved in liquid nitrogen and immediately stored at –80° C until DNA extraction.

Preparation of genomic DNA (Adapted from Perchuck, 2000)

Cryopreserved *R. sylvatica* tadpoles were taken from –80° C and immediately the tails were removed and homogenized in 2 ml of pre-warmed (55° C) extraction buffer (50 mM Tris, pH 8.0, 0.1 M NaCl, 0.1 M EDTA, 0.5% SDS, Proteinase K, added to a final concentration of 50µg/ml immediately before use). Samples were incubated at 55 °C for at least 60 minutes. 2 ml of equilibrated phenol was added and samples were rocked for 30 minutes. Phases were separated by centrifugation and the aqueous phase was removed, combined with 2 ml of phenol/chloroform 1:1, and rocked for 30 minutes. Phases were separated by centrifugation and the aqueous phase removed and combined with 2 ml of ice cold 100% ethanol. Samples were left at -20° C for 1 hour to overnight. DNA was collected by centrifugation and the pellet was air dried. DNA was then dissolved in 2 ml of resuspension buffer [TE (10 mM Tris pH 8.0, 1 mM EDTA) with 0.7 M NaCl, 0.1 M Tris pH 8.0, and 1/9 volume of 10% Cetyltrimethlammonium bromide in 0.7 M NaCl]. 2 ml of chloroform was then added and samples were rocked for 10 minutes. Phases were separated by centrifugation. The aqueous phase was removed and

combined with 4 ml of ice cold ethanol and left at –20° C for 1 hour to overnight. DNA was collected by centrifugation and pellet was air dried and resuspended in 75-100 µl TE buffer.

Genomic Restriction Digests

All restrictions were done using Sau3AI restriction endonuclease (New England Biolabs). Digests were comprised of 50 ul of genomic DNA, 7µl appropriate 10X buffer, 7µl 10X BSA (NEB), and 5µl Sau3AI. Digest reactions were incubated at 37° C overnight.

Agarose Gel Electrophoresis

Nu-Sieve (BMA) agarose and 1X TAE buffer (0.04 M Tris-acetate, 2 mM EDTA) pH 8.5 were used in the preparation of gels. All gels were 2% agarose. To digested DNA samples 10X loading buffer (20% Ficoll 400, 0.1 M EDTA, 1% SDS, 0.25% bromphenol blue, 0.25% xylene cyanol) was added to a final concentration of 2X prior to loading the gel. Promega 1000 bp DNA ladder was run in duplicate on all gels (5 µl/lane). Gels were run with 1X TAE buffer at 5-10 volts/cm between electrodes until the bromphenol blue approached the end of the gel.

Southern Blot Transfer

Following electrophoresis, gels were incubated at room temperature in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes with gentle shaking. A second incubation at room temperature in transfer solution (8 mM NaOH, 3 M NaCl) was also

done for 30 minute with gentle shaking. DNA was then transferred to a Biodyne B positively charged nylon membrane (Pall Gelman Laboratory) using a standard upward capillary transfer method. Gels were left to transfer overnight. Following transfer, membranes were washed in 2X SET (20X SET: 3 M NaCl, 0.6 M Tris, 40 mM EDTA, pH 8.0) for 5 minutes and allowed to dry. DNA was then fixed to membranes by means of ultraviolet cross-linking.

Probe Labeling

The pPVr 9.8 ribosomal probe (Quiel, 1996; Schlesinger, 1999) was used in all hybridization experiments. Probe was labeled using alpha-^{32}P dCTP (3000 Ci/mM) (ICN) and the Prime-It® Random Primer Labeling kit (Stratagene). Newly labeled probe was purified using Ambion NucAway spin columns. Probe activity was quantified (in cpm/µl probe) using Ecoscint O scintillation solution in a Beckman scintillation counter.

Hybridization and Signal Detection

Membranes were pre-hybridized in 30 ml Church hybridization buffer (1% BSA, 1 mM EDTA, 0.5 M Na_2HPO_4/NaH_2PO_4 pH 7.2, 7% SDS) (Church & Gilbert, 1984) in 38 mm x 300 mm hybridization bottles at 55 °C in a rotating hybridization oven for at least 60 minutes. Probe was added at a concentration of $1x10^6$ cmp/ml hybridization buffer and allowed to incubate in the 55 °C rotating oven overnight.

All washes were done at 55 °C in the same hybridization bottles and oven. There were 4 washes total and were done as follows:

1) 5 minute wash with 2X SSC
2) 5 minute wash with 2X SSC
3) 30 minute wash with 2X SSC + 1% SDS
4) 30 minute wash with 1X SSC

Membranes were then placed on filter paper moistened with 1X SSC, wrapped tightly in cellophane and exposed to Kodak BioMax MS film with an intensifying screen at –80° C for 2-48 hours. Films were developed according to standard protocols.

Band detection and normalization of migration distance

Developed films were scanned and imported into Adobe Photoshop version 5.5 and migration distances of bands were calculated to the nearest 0.1 mm using the measure tool. A band of approximately 480 bp was observed to be completely non-polymorphic, appearing in every sample within this study as well as all preliminary study samples. Within each individual the migration of every other band was normalized to the distance of the 480 bp fragment. The 480 bp band was then left out of the subsequent analysis, neither scored as shared nor distinct between individuals. Relative migration distances (RF) for normalized bands were calculated using Microsoft Excel.

A mean and 95% confidence interval was calculated for RF of the 500 bp fragment of the DNA ladder across all gels (N = 14). This confidence interval, +/- 0.02, was used to score bands as shared between individuals.

Determination of Experimental Groups

Experimental groups were chosen by randomly pairing tadpoles from egg masses collected from pools that fit the comparison criteria. Table 1 shows how individual tadpoles were compared to compile our experimental groups.

Table 1. Comparisons used to make experimental groups

Group	Comparison made
Sibling	2 tadpoles from the same egg mass
Pool	2 tadpoles from different egg masses within a pool
Close	A tadpole from pool L5 to a tadpole from pool L6
Far	A tadpole from pool L5 or L6 to a tadpole from pool L1
Distant	A tadpole from pool L5, L6 or L1 to a tadpole from pool U1

Samples from all pools contained tadpoles from two different egg masses, except for pool L6, in which one egg mass experienced 100% mortality. When comparisons were made between pools with samples from two different egg masses, the random pairing was stratified to have an equal number of pairs chosen from each possible combination of egg masses. Individuals were paired with the goal of a sample size of 40 in each experimental group.

Calculation of similarity index (*S*)

A similarity index (*S*) between two individuals was calculated as $S = (2m_{ab}) / (n_a + n_b)$, where m_{ab} is the number of bands shared between individuals a and b, and n_a and n_b are the number of bands observed in individuals a and b. Bands were scored as shared when there was an overlap of the previously described RF confidence interval (0.02). An *S* of 1.0 would indicate two individuals who possess identical

fingerprints, while an *S* of 0.0 would represent two individuals with no bands in common.

Statistical Analyses

SPSS (version 11.5) was used for all analyses. Arcsin ($\sqrt{S}$) was used to transform *S* values between individuals before proceeding with analyses. A one-way analysis of variance (ANOVA) was performed to detect if there was a significant difference between groups. Orthogonal contrasts were decided *a priori* to test differences between selected combinations of groups (Table 2). An α value of 0.05 was used to reject all null hypotheses.

Table 2- Contrast Coefficient table

Contrast	Experimental Group				
	Sibling	**Pool**	**Close**	**Far**	**Distant**
1- Sibling vs. non-sibling	-4	1	1	1	1
2- Pool vs. close	0	1	-1	0	0
3- Close vs. far	0	0	1	-1	0
4- Close & far vs. distant	0	0	1	1	-2

RESULTS

Using the restriction enzyme Sau3AI and the pPVr 9.8 ribosomal probe we successfully generated DNA fingerprints for individuals from all pools examined in this study. The number of individuals from each egg mass for which we produced a readable fingerprint, and we therefore included in our analysis, is presented in table 3.

Table 3- Number of fingerprints analyzed from each sample egg mass

Egg mass (pool name-egg mass #) L=Lincoln Woods, U= Upton	**Number of individuals successfully fingerprinted**
L1-1	12
L1-2	17
L5-1	19
L5-2	19
L6-1	19
U1-1	19
U1-2	20

Fingerprints were found to contain between six and thirteen bands ranging from approximately 2.0 to 0.2 kilobases (Fig. 4) Polymorphisms were apparent throughout this fragment size range. Since fragments smaller than 200 bp did not appear to hybridize reliably, only bands larger than 200 bp were included in RFLP analysis.

Using RF values for each band and an error determined by comparison to the 500 bp band within the DNA ladder, similarity indices (*S*) between individuals within each comparison group was determined as described previously. *S* values calculated for each group are summarized in figure 5.

Figure 4- A representative set of DNA fingerprints from pool L5, egg mass 2. Lanes 2 through 21 each contain genomic DNA from an individual tadpole, cut with Sau3AI, and probed with the pPVr 9.8 ribosomal probe. Lanes 1 and 22 contain 5 μl of a 100 bp DNA ladder.

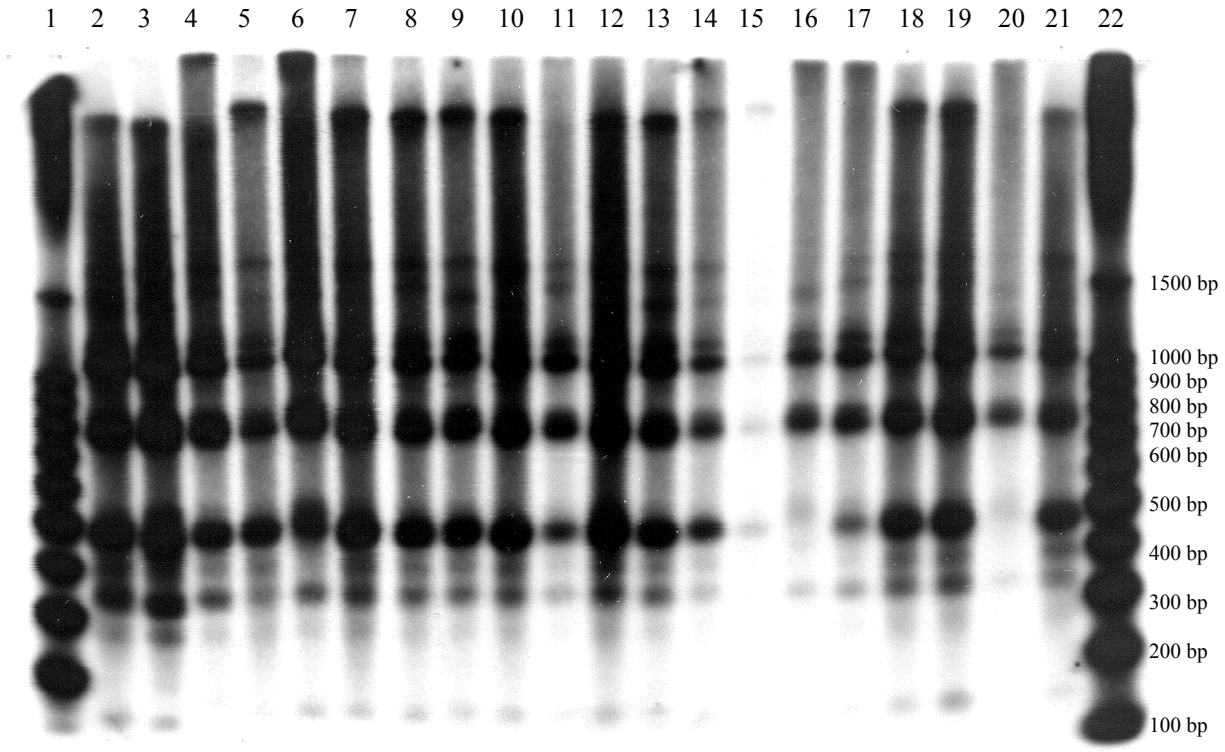
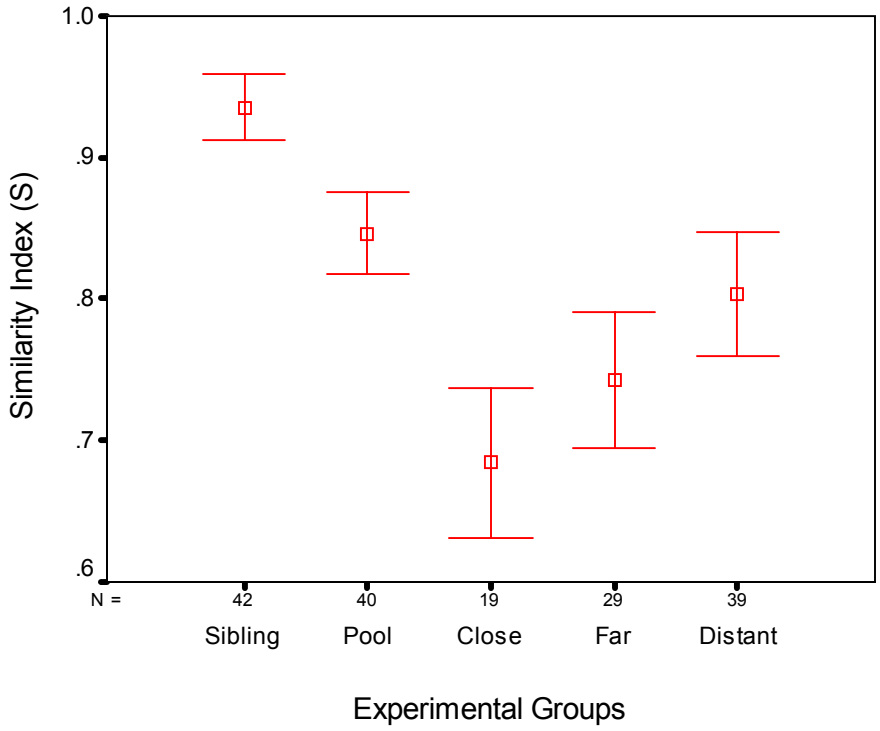


Figure 5. Means plot with 95% confidence interval of similarity index *(S)* for experimental groups.



As shown in figure 5, these data indicate that while the sibling group was clearly the most related, the lowest similarity index was found in the close pool comparison.

An analysis of variance (ANOVA) performed on the transformed data showed a highly significant difference among groups ($F_{4,168} = 28.7$, $p < 0.001$) (Table 4). Of the four previously described contrasts performed three were found to be significant ($p \leq 0.001$) (Table 5), with the only non-significant contrast being the comparison of the close and far groups ($p = .074$). All contrasts were done assuming unequal variance among groups (Levene's Statistic = 5.561, $p < 0.001$).

Table 4. ANOVA table for relatedness of tadpoles

	Sum of squares	df	Mean square	F	Sig.
Between groups	3.009	4	.775	28.713	*<0.001*
Within groups	4.425	164	.027		
Total	7.524	168			

Table 5. Contrast output with 2-tailed sig. values (α = 0.05). All contrasts were done assuming unequal variances.

Contrast	Value of contrast	Standard error	t	df	Sig. (2-tailed)
1- Sibling vs. non-sibling	-1.1942	.12799	-9.331	59.713	*<0.001*
2- Pool vs. close	.2006	.03339	6.007	34.285	*<0.001*
3- Close vs. far	-.0721	.03941	-1.830	43.889	0.074
4- Close & far vs. distant	-.2579	.07572	-3.406	63.852	*0.001*

Contrast 1 – Sibling vs. Non-sibling

Our first analysis compared the relatedness between siblings (individuals from the same egg mass, which could be full- or half-siblings) to all other groups. As expected, siblings were the most related group in our analysis, ($t = -9.331$, $p < 0.001$) with an *S* of 0.936.

Contrast 2 – Pool vs. Close

We next examined the relatedness between individuals from close pools as compared to that between individuals from the same pool, but different egg masses. The data indicated that individuals from close pools are less related to one another than non-sibling individuals from the same pool ($t = 6.007$, $p < 0.001$). The similarity indices for the pool and close groups are 0.846 and 0.684 respectively.

Contrast 3 – Close vs. Far

An examination of the relatedness of individuals from close pools as compared to far pools suggests no significant difference. ($t = -1.803$, $p = 0.074$). The *S* values for the close and far groups are 0.684 and 0.743 respectively. The lack of a significant difference between these groups is somewhat unexpected considering our initial prediction that genetic similarity should decrease as distance between groups increases.

Contrast 4 - Close & Far vs. Distant

The final analysis compared the relatedness of the Lincoln Woods pools to the relatedness between Lincoln Woods pools and the Upton pool. Again, the somewhat unexpected result was that, when looked at collectively, the Lincoln Woods pools appear more related to the geographically isolated Upton population, than they are to each other ($t = -3.406$, $p = 0.001$).

We initially predicted that as we compared *R. sylvatica* populations over increasing spatial distance we would see a drop in the genetic similarity between them (*i.e. S* should decrease). The results of previous studies as well the connection between dispersal and gene flow led us to adopt this prediction. Additional findings in published works, noting the keen homing ability of the woodfrog to return to its natal pool in addition to its relatively large home range with respect to our study area, also contributed to our assumption that pools in close proximity to one another should show very little, if any, genetic variation. While we demonstrate that over a distance of about two hundred meters there is sufficient gene flow to cause populations to appear genetically similar

(contrast 3, close vs. far), our data also suggest that genetic distinction can arise between pools separated by only a few meters (contrast 2, pool vs. close). This is an interesting finding in that no one has found genetic differences in wood frogs on such a small spatial scale. We propose that some of our unexpected results are due to wood frogs selecting breeding sites based on information beyond simply proximity to their natal pool.

DISCUSSION

This study used RFLP analysis to assess the genetic relatedness of *R. sylvatica* populations found within a vernal pool array. Genetic differentiation between fragmented species groups has been used to estimate population parameters such as dispersal, gene flow and genetic drift (Gibbs, 1998; Newman & Squire, 2001; Shaffer *et al.*, 2000, Vos *et al.*, 2001). These population estimates can assist conservation biologists in making informed management decisions regarding fragmented populations of a species. The comparison groups used in this study were chosen to allow observation of genetic relatedness over very small (within the same pool) to relatively large (40 km) ranges. Average relatedness among siblings within egg masses was also calculated to establish a baseline for maximum genetic sharing. We predicted that as we made comparisons over an increasing spatial scale, we would see a decrease in genetic similarity between groups, relative to sibling relatedness. This prediction was based on a number of previous studies that suggest amphibian populations should display greater genetic isolation from one another as the distance between them increases (Berven & Grudzien, 1990; Gibbs, 1998; Newman & Squire, 2001). Our data do not fully support this prediction. While there were clear differences in relatedness across comparisons, we did not observe a monotonic decline in relatedness as distance between experimental groups increased.

Our finding that siblings are more closely related than any of our other experimental groups (contrast 1) is consistent with our predictions. Individuals from within the same egg mass should display the highest amount of genetic relatedness to one another since they are either full- or half-siblings. Had we found sibling relatedness to be

equal to that of "close" or "far" pools it would imply a panmictic genetic structure with all groups freely interbreeding. However, if sibling relatedness did not differ from relatedness to a "distant" population, it would suggest that the molecular techniques used in our analysis were not sensitive enough to detect genetic differences in *R. sylvatica.* Sibling relatedness served as our positive control, so once it was found to be distinct it was not included in subsequent contrasts.

Individuals from close pools (L5 and L6), separated by only 5 m, were found to be less related than non-sibling individuals from the same pool (contrast 2). This is an interesting finding because it shows genetic differentiation between woodfrog populations separated by only a few meters, a smaller scale than found in previous studies. Wood frogs have been characterized as highly philopatric, using celestial, visual and olfactory cues to home in on their natal pool (Berven & Grudzien, 1990). Our data indicate that even when vernal pools are in close proximity to one another, as is the case with L5 and L6, individuals will not casually switch to a neighboring pool.

Further examination of the ecology of pools L5 and L6 revealed possible reasons for this. The characteristics of pool L6 fit the definition of a vernal pool more closely than L5, although both are certified as vernal pools. One of the visible differences between L5 and L6 is the species they support. Pool L6 supports populations of clam shrimp, fingernail clams and snails, which L5 does not. However, pool L5 hosts a greater diversity of dragonfly species as well as mayflies (Colburn, personal communication, 2003) (order Ephemeroptera) and green frogs (Robakiewicz, personal communication, 2003), *Rana clamitans*, two species absent from pool L6. The differential presence of

these species can give insight into the inherent differences between the pools that individuals may be using to make breeding site choices.

Green frogs, unlike the wood frog, can overwinter in pools as larvae and complete metamorphosis during their second season. The presence of a population larger, year-old green frog tadpoles in a pool could be seen as a competitive pressure against smaller, newly hatched, wood frogs tadpoles. Wood frogs that can assess the differential presence of a competitor, like green frog larvae, between two possible breeding sites may choose to have their offspring in the less competitive environment. Also individuals adapted to breeding in the absence of certain competitors may not casually switch over and breed in a nearby pool with increased competitive pressures.

Another interesting difference between pools L5 and L6 is their pH. Over a number of seasons pool L6 consistently measured 0.5 to 1.0 pH units higher than pool L5 (Colburn, personal communication, unpublished data, 2003). The more alkaline nature of pool L6 would support the presence of snails and other mollusks not found in pool L5. The effects of acidity on amphibian embryonic and larval development has been well studied and can impact sperm activity and hatching success, as well as increase embryonic mortality and reduce larval development rate (Cummins, 1989; Gosner & Black, 1957). A reduction in larval growth rate can also increase the risk of predation as well as increase mortality due to the drying of ephemeral habitats (Cummins, 1989). Groups that are found in more acidic environments have most likely adapted metabolic and developmental mechanisms to deal with a lower pH. However, the offspring of individuals reared in more alkaline pools (L6) who attempt to breed in an acidic pool (L5) may experience a strong selective disadvantage.

While we note that species assemblages and chemical properties differ between these geographically close pools, we do not propose these are solely responsible for the difference in relatedness between woodfrog populations in these pools. We do suggest, however, that wood frogs may choose mating pools according to characteristics other than proximity to their natal site. This is further support for preserving each pool as an individual population since there may be characteristics unique to a pool that will keep residents from breeding in a neighboring site simply because it is close by.

The relatedness between close pools was not found to differ statistically from the relatedness of far pools (contrast 3). This is extremely informative because while we have demonstrated genetic differences over a relatively small distance (~ 5 m), there appears to be sufficient gene flow to make distinction less obvious over a larger distance (~ 200 m). Again, in contrast to previous studies, this finding supports the idea that genetic differentiation between populations is dependent on factors other than distance. The data suggest that a post-hoc analysis of the specific relatedness of pool L1 to L5, and pool L1 to L6, may be very informative. However these comparisons were not part of the original experimental design and we do not feel that it would be prudent to proceed and conduct those tests at this time.

Another important observation in this regard was that genetic relatedness among the three pools of the Lincoln Woods sanctuary (L1, L5 & L6) was lower than that between the Lincoln Woods and Upton pools (contrast 4). One would expect the comparison of individuals from the Leominster and Upton sites to be representative of any random pairing of *R. sylvatica* from geographically separate populations. Again, we propose that the observed decrease in similarity between the Lincoln Woods pools is due

to habitat- and site-specific characteristics of the sanctuary, such as those of pools L5 and L6. Such fine-level discrimination in breeding site choice would not be apparent in the Leominster-Upton comparison since individual frogs clearly cannot directly compare between these two geographically distant sites.

Explanations of differences between pools L5 and L6, based on variable pool characteristics, have been proposed above. We suggest that in addition the overall differences observed among the Lincoln Woods pools is best described by the heterogeneous landscape of the sanctuary. For an area of only 68 acres, Lincoln Woods sanctuary is quite topographically dynamic, not unlike many New England forests. We feel that this landscape heterogeneity would help explain some of the relatively small-scale trends we document, which are in contrast to some previously published works. For example, the study done by Newman and Squire using microsatellite analysis (2001) was conducted over a series of pools from the Great Plains region of North Dakota. The landscape of an area of the Great Plains is likely to appear much more homogeneous than a similar square area in a New England forest. Over the relatively homogeneous Great Plains landscape, there may be fewer variables present to differentiate one breeding site from another, therefore resulting in broader, more monotonic dispersal patterns for species like *R. sylvatica*. This factor alone may explain why studies done over these homogeneous landscapes correlate genetic differentiation closely with distance, in addition to finding differences only over the largest spatial separations (well over one kilometer). A study by Vos *et al.* (2001) on the genetic similarity of fragmented populations of the moor frog, *Rana arvalis*, found that the presence of physical barriers to dispersal (roads and railroad tracks) had a more pronounced effect on genetic divergence

between populations than did the distance separating fragments. This study suggests that as barriers introduce an element of heterogeneity to a landscape, variability in the landscape exerts greater influence on dispersal and genetic structure than does spatial separation alone.

Implications for management.

When drafting management plans it is important to attempt to preserve as much genetic variation within a species as possible. For species that breed in isolated habitats, such as the wood frog, estimates of the relatedness between neighboring populations can assist conservation biologists in deciding whether to preserve some or all groups in a management area. Previous studies have suggested that all vernal pools within a one-kilometer radius should show little, if any, divergence in the genetic structure of *R. sylvatica* populations they host (Berven & Grudzien, 1990; Newman & Squire, 2001). This would imply that, with respect to *R. sylvatica*, overall genetic diversity would not be lost if two of five vernal pools were allowed to be destroyed within a proposed development site with a one kilometer radius or smaller.

Our study proposes that relatedness between populations of *R. sylvatica* within a spatially heterogeneous series of vernal pools is based on the physical characteristics of their breeding pools as well as the landscape structure between them. Making inferences of relatedness between pools based only on the distance between them may overestimate similarity and lead to management decisions that result in the loss of genetically unique populations. Ideally, until more concrete generalizations of wood frog genetic structure are made, one should perform genetic analysis on the populations in question to determine relatedness. We realize this may be impractical due to the time and cost of such a study, however at a minimum we suggest looking at parameters beyond spatial separation when assessing similarity of habitats and populations. Also, assuming that all certified vernal pools within a relatively small geographical range equivalently meet the needs of obligate breeders may lead to poor management decisions. We believe that

many pools that appear very similar may actually offer very different breeding habitats to organisms such as the wood frog. Natural histories of the pools in question as well as landscape characteristics such as dispersal barriers and corridors may all influence genetic diversity of neighboring populations.

Maintaining high levels of genetic diversity will help species survive catastrophic events such as climatic variation, introduction of invasive exotics, disease or pollution. Management decisions based on the assumption that all *R. sylvatica* populations in close proximity to one another are genetically similar could allow for rare alleles to be lost due to habitat destruction. Following catastrophic events that greatly reduce population size it is often individuals possessing these rare alleles that are able to reestablish extirpated populations that were lacking the genetic capability to adapt to their new environment. For this reason we propose management decisions for the vernal pool breeder *R. sylvatica* be made on a pool-by-pool basis. By taking into account more than spatial distribution of neighboring pools, ecological managers should more effectively maximize overall genetic diversity of wood frog populations.

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