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Genetic Population Structure of the Plains Spadefoot Toad (Scaphiopus (Spea) bombifrons) On the Northern Plains

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GENETIC POPULATION STRUCTURE OF THE PLAINS SPADEFOOT TOAD (*Scaphiopus (Spea) bombifrons*) ON THE NORTHERN PLAINS.

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This thesis, submitted by Todd C. Becker in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Robert A. Newman
Chairperson

This thesis meets the standards for appearance, conforms to the style and format requirements of the Graduate School of the University Of North Dakota, and is hereby approved.

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ABSTRACT

In this study, I examined genetic population structure in spadefoot toads (*Scaphiopus bombifrons*) living in western North Dakota. Spadefoot toads have a very unique life history among amphibians. They are generally limited to being active only during nocturnal wet periods. Because of this, and other factors such as philopatry, it is expected that migration and dispersal rates should be relatively low. I hypothesize that this low rate of movement, along with fairly short dispersal distances when individuals do move, should lead to the structuring of genetic variation at relatively fine scales.

Five microsatellite loci were used to examine population structure at 9 temporary breeding ponds. Distances between ponds ranged from <500m to 14 km. The data showed evidence of significant genetic variation occurring between ponds even at the shortest interpond distances.

A strong heterozygote deficiency was observed for 4 out of the 5 microsatellite loci, suggesting that inbreeding may be occurring in the system. A pattern of isolation by distance was also observed but was not statistically significant. High Fst and Rst values at short interpond distances deviated from the pattern predicted by isolation by distance, and may be driven by stochastic processes such as drift, bottlenecks, and founder events.

CHAPTER I

INTRODUCTION

Population Genetic Structure

The genetic structure of a population refers to how genetic variation is distributed throughout a single population or a collection of interconnected local populations. The present genetic structure of a population is shaped by demographic, genetic, and historical factors (Slatkin 1994). It is important to understand the genetic structure of populations and what forces are playing an important role in shaping that structure, because of the implications for understanding phenomenon such as the process of speciation (Barber 1999, Harrison and Hastings 1996). The pattern of the structuring of genetic variation also gives insights into what is a relevant biological scale for a given species. This issue of biologically relevant scale also plays an important role in determining a successful approach for the current management of a given species especially if one of the goals is to maintain genetic diversity (Haig 1998; Seppa and Laurila 1999).

Differing levels of drift, selection, mutation and gene flow can give rise to a wide array of potential genetic structures in a given population. These forces can be assigned to two main categories based on their overall effect on genetic diversity. The first group includes mutation and gene flow, which are generally viewed as adding to the genetic variation present within a population. They also have an opposite effect on genetic

differentiation within populations by acting to 'tie' populations together. Genetic drift and selection on the other hand tend to reduce overall levels of genetic variation, but generally increase genetic differentiation and can cause local populations to diverge from each other over time.

Mutation is the ultimate source of new genetic variation. Recombination also plays a role in generating variation in quantitative genetic traits by rearranging existing gene complexes, but mutation is still the ultimate source for all new genetic variation. Gene flow adds to genetic diversity within populations by spreading out and maintaining variation that is created by mutation. This has the net effect of tying populations together genetically and prevents the divergence of local populations due to the effects of drift and selection. This also leads to local populations that have overall lower levels of genetic differentiation.

This study is primarily concerned with neutral genetic variation that is for the most part unaffected by selection. Measuring genetic variation with neutral markers eliminates selection as a confounding factor when examining the effects of the remaining genetic forces on the study populations. A selectively neutral marker with a relatively high mutation rate such as microsatellites seemed well suited for examining genetic variation at a fine spatial scale, and will be discussed later.

Although selection and mutation are very important evolutionary forces, especially over large spatial and temporal scales, the structuring of genetic variation at the local level is primarily the result of gene flow and drift (Slatkin 1993). This is because as geographic distance between populations becomes smaller, migration between

local populations becomes more frequent leading to an increase in the exchange of genetic material between the populations.

If populations examined on a fine spatial scale are small, semi-isolated local populations, drift becomes increasingly important because of the smaller effective census size of these populations. Small populations are more vulnerable to the effects of demographic and environmental stochasticity, which reduces effective population size and makes events such as founder events and bottlenecks more common. All of these factors act to greatly magnify the effects of drift at fine spatial scales, and could lead to the structuring of genetic variation at very fine scales. Because the focus of this study is the fine scale structuring of genetic variation, the remainder of this discussion will focus on the effects of drift and gene flow in shaping the distribution of genetic variation at the local population level.

Genetic drift was first described by Wright (1931) as a shift in gene frequency due to chance, which can occur in the absence of selection, migration, or mutation. Genetic drift is generally regarded as a force that reduces the overall genetic diversity within a population by driving individual alleles either to fixation or extinction. Of course the relative strength of drift depends on the size of the population as well as the magnitude of other forces acting on the population such as gene flow, selection and mutation. It should also be noted that drift can also potentially play a role in facilitating genetic diversity as outlined in Wright's Shifting Balance theory of evolution (Wright 1969).

The effect of drift is greater in smaller populations because these populations have a smaller effective population size (*Ne*) (Wright 1931). Effective population size is a calibration number representing the size that an ideal population would have to be to have

the same genetic characteristics that are found in the real world population. An ideal population in this context is one that meets all the assumptions of Hardy-Weinberg, as well as the assumption that the number of males and females are equal, and that each individual has an equal chance of contributing gametes to the next generation (Hartl 1980). Therefore, populations with smaller *Ne* will have fewer individuals contributing gametes to the next generation, and a larger probability of a random shift in gene frequencies due to sampling error.

It has also been recognized that drift is more likely to occur in populations that are subdivided into semi-isolated local populations, because the effective population size of the series of local populations is smaller than that of a single panmictic population made up of the same number of individuals (Wright 1938; Gilpin 1991). This makes the genetic subdivision of populations a potentially important factor in evolution by promoting genetic differentiation.

Gene flow refers to the exchange of genetic material between two populations. Gene flow is generally viewed as a homogenizing force that counteracts the diversifying effects of drift and selection by spreading genetic variation between populations. This tends to reduce the structuring of genetic variation, and in local populations connected by fairly high rates of gene flow it gives rise to populations that are more similar in genetic composition.

The level of gene flow needed to counteract the effects of drift and selection depends on the relative magnitude of these forces. Wright (1931) first showed that the exchange of an average of as little as one or more individuals between two populations could be enough to prevent different alleles at a neutral locus from being fixed in the two

populations. This result is also independent of population size because in larger populations the effect of drift is weaker and therefore fewer migrants (as a fraction of the total size of the population) are required to keep populations from drifting apart (Slatkin 1987).

Experimental Hypotheses

The object of this study is to examine the distribution of genetic variation in populations of plains spadefoot toads (*Scaphiopus bombifrons*) in western North Dakota. Other studies have shown that amphibian populations tend to be highly structured genetically (Shaffer et al. 2000) due to their low mobility, but at what scale? I hypothesize that because of their unique life history and the harsh environment in which they live, spadefoot toads will show significant genetic differentiation even at very fine scales.

Spadefoot toads were chosen because of their unique life history. Low mobility due to the arid environments in which they live, coupled with the fact that they spend much of their time under ground decreases their dispersal capability and therefore likely leads to fairly small amounts of gene flow between local populations. This in turn would likely lead to a high degree of genetic structuring among local populations, perhaps even at very fine spatial scales. An understanding of what spatial scales are biologically relevant to a species can play an important role in

In order to measure genetic structure at the fine spatial scales that were of interest to this study, a selectively neutral marker with a relatively high level of polymorphism had to be chosen. Microsatellite DNA sequences are well suited to measure genetic differentiation at a fine scale because of two important attributes. The first is their high

mutation rate (leading to a high level of polymorphism) and second they are selectively neutral. Microsatellite structure, formation, and mutational characteristics will be discussed in the next section.

Microsatellites

Microsatellites are sequences of very short, tandemly repeated nucleotide motifs (e.g. AAT) that occur throughout the genomes of all eukaryotes (Schlotterer and Tautz 1992). Studies of the human and mouse genomes have shown that these sequences are generally distributed throughout the genome; with the exception of the regions around the telomeres where repetitive sequences are observed, but occur at a lower density (Dietrich et al. 1996; Dib et al. 1996). These sequences are known to be highly polymorphic due to their high mutation rate, making them well suited as genetic markers to be used for identity testing, population studies, linkage analysis, and genome mapping (Tautz 1989).

Two different models have been proposed to explain the mechanism causing the observed instability and high mutation rate in microsatellites; these are slip strand mispairing (SSM) and unequal crossing over (UCO) (Wierdl et al. 1997; Eisen 1999). Unequal crossing over occurs when homologous chromosomes become misaligned during recombination, this is thought to occur more frequently in areas containing microsatellites because repetitive sequences increase the likelihood of misalignment (Eisen 1999). Slip strand mispairing occurs when the DNA polymerase 'slips' during DNA replication causing the template and newly synthesized DNA strands to become briefly disassociated. When the two strands re-associate they may become temporarily unaligned causing the formation of a loop of unpaired DNA. This loop is generally composed of one or more repeat units, which protrude out from one of the two strands

effectively shortening the strand containing the loop. If DNA replication continues before the loop is repaired, the newly synthesized strand will change in length by the number of base pairs (usually a multiple of the repeat unit length) contained in the loop. If the loop is contained on the synthesized strand then this strand will increase in length, if it is on the template strand then the new strand of DNA will decrease in length (Wierdl et al. 1997; Eisen 1999)

Several studies, shown below, illustrate that slip strand mispairing plays a much larger role in causing microsatellite instability than does unequal crossing over. If unequal crossing over played a major role in creating the high mutation rate seen in microsatellites then it would be expected that mutations that interfere with recombination would lower the mutation rate. Levinson and Gutman (1987) found that microsatellite stability was unaffected by mutations in the recA gene in *Escherichia coli*, which plays an important role in recombination, suggesting that unequal crossing over does not play a great role in determining microsatellite mutation rates. They also found that mutations in genes controlling mismatch repair caused stability of microsatellite sequences to be greatly reduced. A similar result was also obtained by Weirdl et al. (1997), suggesting that the mismatch repair system plays an important role in correcting replication errors caused by DNA polymerase slippage and stand misalignment, which would otherwise result in mutations in the microsatellite sequence. Thus, the mutation rate at microsatellite loci is the result of a balance between the generation of mutations, primarily by slip strand mispairing, and the correction of some of these errors by exonucleolytic proofreading and mismatch repair (Eisen 1999).

Mutation rates in microsatellites have been estimated to range from 1.2×10^{-4} to 1.5×10^{-2} per base pair. This is several orders of magnitude higher than the mutation rates at other loci in the same genome, and it should also be noted that in some taxa the rate is much lower (Eisen 1999; Jin et al. 1996).

Jin et al. (1996) observed that the mutation rate at a single human microsatellite locus varied among alleles. Specifically, alleles with greater length tended to have a higher rate of mutation than shorter alleles. Weirdl et al. (1997) detected a similar pattern in *Saccharomyces cerevisiae*, microsatellites with longer tract lengths had a much higher rate of instability. They observed that microsatellite instability was 500 fold greater for a 105-bp repeat tract than for a 15-bp tract. This pattern of increasing instability within a repeat region as length increases agrees well with the predictions of the slip strand mispairing model, a higher mutation rate is expected in longer repeats because there are more chances both for strand slippage and for strand misalignment (Eisen 1999).

This property of microsatellites, which gives rise to their high mutation rates and leads to high levels of polymorphism, makes these markers particularly amenable for use in studying the genetic structure of populations especially at relatively fine spatial and temporal scales (Estoup et al. 1998). In particular, the high level of variation at microsatellite loci suggests that these markers may be more sensitive to breeding population size, population structure, and rates of dispersal than other widely used nuclear markers such as allozymes (Scribner et al. 1994; Estoup et al. 1998; Parker et al. 1998). The fact that these markers are co-dominant also allows investigators to examine levels of heterozygosity and the distribution of genotypes within populations, allowing for comparison with Hardy-Weinberg equilibrium predictions.

Microsatellite polymorphism also appears in most cases to be selectively neutral. Selective neutrality cannot necessarily be assumed for all allozyme loci because differences between populations may reflect adaptation to local environment (Estoup et al. 1998). Clinal geographic variation in allozyme frequencies due to natural selection has been previously observed (e.g. Bergmann 1975, 1978), and microhabitat specialization by electrophoretically distinct genotypes has also been reported (Hamrick and Allard 1972, Heywood and Levin 1985). These studies suggest that selection can act directly on allozymes or on traits to which they are genetically linked. Selection can also act to maintain genetic variation itself due to balancing selection promoting the persistence of genetic polymorphisms (Parker et al. 1998).

The above outlined attributes illustrate why microsatellite markers were chosen for this particular study; specifically, their high level of polymorphism and selective neutrality. This allowed for a high degree of resolution of the genetic structure present in a population, even between sample locations that were less than one kilometer apart. The relatively high level of polymorphism at any one locus also allowed a fairly minimal number of loci to be used (five in this case), which reduced the amount of time and effort necessary to produce genotypes for each sampled individual.

Spadefoot Life History

The organism selected for this study, *Scaphiopus bombifrons*, was chosen because of its unique life history, and the effect that this life history will likely have on the structuring of its genetic variation. I predict that as a result of adaptations that the spadefoot has developed in order for it to survive in an arid climate; genetic variation will show detectible structure, even at fine spatial scales. As spadefoot life history is

discussed, special emphasis will be placed on examining characteristics of its habits that could play an important role in the structuring of genetic variation.

The plains spadefoot toad, *Scaphiopus bombifrons*, is widely distributed throughout the central plains of the United States and southern Canada. At the northern extreme of its known range this species occurs in the grasslands of southern Alberta, southern Saskatchewan, and southwestern Manitoba (Klassen 1998; Lauzon and Balagus 1998). It is also found continuously throughout the central Great Plains of the U.S. from eastern Montana and western North Dakota in the north, to eastern New Mexico, west Texas, and northern areas of Mexico in the south. There are also small isolated populations in extreme southern Texas and adjacent areas of northern Mexico. The eastern extreme of its range extends into central Missouri and eastern Oklahoma (Stebbins 1951, 1954).

Plains spadefoots generally range from 3.7 to 6.3 cm in length, are stout bodied, and have relatively smooth skin. The skin ranges in color from dark brown to gray except on the ventral side where it is white. The back usually has several barely discernable light stripes, and is flecked with orange to yellow tubercles (Stebbins 1951, 1954). The presence of a prominent 'boss' or bump between the eyes, vertically oriented pupils, and the single, black, sharp edged metatarsal tubercle distinguish the plains spadefoot from other members of this genus (Stebbins 1951).

The plains spadefoot inhabits plains, hills and river bottoms in mixed grass prairie, sagebrush habitats, desert grassland, and farmland in regions of low rainfall. They prefer areas with loose, sandy or gravelly soil that is suitable for burrowing (Stebbins 1951; Wright and Wright 1949). Spadefoots form their burrows by using the

hard, sharp-edged tubercle or 'spade' on the inner surface of the hind foot to push aside soil as they back into the ground. The burrows of adult toads range in depth from several inches to several feet (Stebbins 1951; Wright and Wright 1949). The depth of the burrow has been observed to be affected by moisture conditions, with animals forming relatively shallow burrows when the soil is moist and deeper burrows as the soil moisture decreases (Bragg 1944, 1965).

All spadefoots are almost completely nocturnal and generally only emerge from their burrows at night to feed during wet weather. Spadefoots have been known to remain in their burrows below the surface for weeks or even months at a time if necessary, only emerging to feed as conditions at the surface become more favorable (Bragg 1965). Adult spadefoots have been observed to feed on various invertebrates including flies, hymenopterans, moths, beetles, bugs, and spiders (Bragg 1944).

Because their surface activities are so greatly restricted, spadefoots are capable of rapidly acquiring and storing energy reserves. Dimmit and Ruibal (1980) calculated that during only two nights of feeding a male Couch's Spadefoot Toad could ingest all the fat necessary to survive for 12 months. Such a short feeding duration would probably not be expected under normal circumstances, but it illustrates how little above ground activity spadefoots can have and still be able to survive. It is this combination of restricting activity almost entirely to periods after sunset, and the ability to excavate burrows and access soil with higher moisture content that allows spadefoots to inhabit such dry environments (Bragg 1965).

Movement patterns of adult spadefoots closely resemble those seen in other amphibians. With the exception of breeding and occasional long distance movement,

adult anurans tend to confine most of their activities to a relatively small home range. Mean home ranges of 64 m^2 (ranged from 2.9-368 m^2) and 60 m^2 (ranged from 20-200 m^2) have been observed in wood frogs and green frogs respectively (Bellis1965; Martof 1953). Pearson (1955) observed spadefoot mean home range sizes of 10.1 m^2 with a range from <1 m^2 to about 83 m^2 in the eastern spadefoot toad *Scaphiopus holbrooki*. It is probable that low frequency longer distance movements may not have been picked up in these studies, but even so these studies suggest that most anurans do not generally move long distances after post-metamorphic dispersal.

Reproduction in spadefoots has evolved to be well adapted to living in an arid environment. Spadefoots lack a well-defined breeding season but rather follow a xeric pattern, characterized by Bragg (1945), in which breeding is initiated by periods of heavy rainfall. Depending on their location, spadefoots may breed at any time between May and August after periods of heavy rain if the air temperature is above about 11° C (Bragg 1945). Spadefoots rely almost exclusively on temporary ponds for breeding. These ponds form after periods of heavy rain in areas such as roadside ditches, drainage basins, and low areas in fields (Hansen 1958; Klassen 1998). Typically, males arrive first at a potential breeding site and begin calling, which eventually attracts females to the area. (Bragg 1945). Egg masses vary in size with larger masses containing 200-250 eggs, while small masses may contain as few as 10-20. These masses are generally attached to submerged vegetation or any other object protruding from the bottom of the pond (Wright 1949).

Tadpoles emerge from eggs after about two days depending on water temperature (Bragg 1965). The rate of larval development is highly variable and is directly related to

water temperature. Metamorphosis occurs anywhere from less than two weeks for Couch's spadefoots (Newman 1989) to about 6 weeks for *S. bombifrons* (Marby and Christiansen 1991). After metamorphosis juvenile toads leave their natal pond and disperse. Dispersal distances for juveniles of the plains spadefoot are not well known, but Klassen (1998) reported observing juvenile spadefoots at locations between 1 and 2.25 km from the nearest known breeding pond. This shows that at least under some circumstances (e.g. favorable weather conditions) the juveniles of this species have the ability to disperse over moderate distances.

Although dispersal distances in plains spadefoots have not been well quantified, the dispersal in other amphibians has been more accurately measured and provides some insight into the frequency and general scale of dispersal for anurans. In a study of post-metamorphic dispersal in Fowler's Toad (*Bufo woodhousei fowleri*), Breden (1987) observed that 27% of the individuals studied bred for the first time in a non-natal pond. He also noted that juveniles are significantly more vagile than adults as shown by the much higher median distance between capture sites. A similar pattern was observed by Berven and Grudzien (1990), in their study of dispersal in wood frogs (*Rana sylvatica*). They reported that none of the 11,195 marked adults migrated from one breeding pond to another. In contrast, they observed that 21% of the marked male and 13% of the marked female juveniles were recaptured as breeding adults at ponds other than those in which they spent their larval periods. Average dispersal distances were 1,140 +/- 324 (SD) meters and 1,276 +/- 435 (SD) meters for male and female metamorphs respectively.

Although adults have been observed to make long distance movements to access breeding ponds, the effect of this movement on gene flow is expected to be relatively

small due to a high degree of breeding pond fidelity which has been observed in many anurans (Bellis1965; Breden 1987; Berven and Grudzien 1990). Because of this and because most of their movement is confined to relatively short distances, it has been observed that the dispersal of juveniles from breeding ponds to areas where they will take up a more permanent residence as adults is responsible for most of the gene flow that occurs between local populations.

The combination of small home range size in adults, breeding pond fidelity, and generally short dispersal distances for juveniles make amphibians a particularly interesting system for examining the distribution of genetic variation. Also, the unique behavioral adaptations that are essential to the plains spadefoots ability to survive and reproduce in arid environments have important implications for the impact of gene flow on population structure. By restricting activity almost exclusively to wet nocturnal periods, the amount of time available for animals to move about is severely limited. Even at night travel over any great distance would be difficult except under very favorable conditions (e.g. after heavy rainfall). All these factors act to restrict the magnitude of migration in spadefoots even more than in other amphibians. This suggests that gene flow between adjacent populations would be relatively low, even if they were not separated by large spatial distances. Therefore, we hypothesize that by employing a sufficiently variable marker, we will see significant differences in genetic structure between spadefoot breeding ponds even at very small spatial distances.

This leads to several predictions that will be tested in the course of this study regarding the structuring of genetic variation in this system. First, because of limited gene flow over even modest spatial distances, differences in allele frequency should

accumulate due to the effects of genetic drift. These changes in allele frequency due to drift will tend to accumulate over time without the homogenizing effects of relatively high levels of gene flow. This will lead to the structuring of genetic variation at relatively small spatial scales. Under this scenario, breeding populations should appear genetically distinct even at modest spatial scales. This differentiation would appear as significant differences in allele frequency at several microsatellite loci between breeding ponds. Secondly, this genetic differentiation should become more pronounced at larger spatial scales due to the reduced importance of gene flow relative to drift at these larger scales. This should lead to an isolation by distance effect that will appear as a positive correlation between genetic distance and spatial distance at the appropriate spatial scale.

CHAPTER II

METHODS

Field Methods

All field sampling took place in western North Dakota, specifically in extreme southwestern portion of Slope County and the western portion of Dunn County (Figure 1). Sampling effort was focused on this region because of prior sightings of plains spadefoots in this area (T. Hoberg, personal communication). All sampled ponds were located in three main areas, two areas in Dunn County and one area in Slope County.

A total of nine breeding ponds were found in the three areas. These ponds had apparently been formed after a series of heavy rains in mid-June 1998. Five of the ponds consisted of shallow drainage ditches along roads that had filled with runoff to create temporary standing water. The remaining ponds were located in low areas in the landscape that had collected runoff to create temporary pools.

All ponds were quite shallow and ranged from approximately 5 cm to 30 cm in depth, with most falling into the 5 cm to 15 cm range. All of the ponds sampled were ephemeral and most had dried by mid July. The water in the ponds was generally quite muddy, often so much so that tadpoles could not be seen despite the shallow depth of the ponds. Vegetation around the ponds was typically sparse and consisted mainly of mixed grasses that often extended into the pond itself and probably provided oviposition sites. No spadefoot tadpoles or adults were seen in any of the permanent or semi-permanent

ponds that were examined. However, tadpoles that appeared to be *Pseudacris triseriata* were commonly seen in these more permanent bodies of water.

The first area to be sampled was in the southwestern portion of Slope County and contained two breeding ponds. Both of these ponds were located in a shallow valley and had apparently formed as a result of the accumulation of runoff from the surrounding hills. Slope County lies in an unglaciated section of western North Dakota and is part of the Missouri River drainage basin. The main drainage system in the area is the Little Missouri River, which receives tributaries from both the east and west. The topography of the area ranges from rolling uplands to highly dissected, erosional badlands. The climate is also semi-arid and is characterized by long cold winters and short warm summers. The mean annual temperature at the nearby town of Marmarth is 5.95° C and the mean annual precipitation is 380 mm (Anna 1981).

The other two areas sampled are located in the central and western portions of Dunn County. The topography of Dunn County varies from gently rolling to highly dissected, and is primarily the result of erosion. The surface substrate in Dunn County is largely glacial till, glaciofluvial sand, and gravel deposits. The climate of the county is cool and semi-arid; the mean annual temperature is about 4.5° C, and the mean annual precipitation in about 419 mm (16.5 inches). About 75% of the annual precipitation occurs in the 6-month period extending from April to September (Klausing 1979).

Sampling was conducted from mid May to late July 1998, with most of the actual sampling taking place between late June and early July. Initial efforts were focused on locating specific areas where spadefoot toads were present. These surveys were conducted following periods of significant rainfall (usually > 2.5 cm), and effort was

concentrated to the hours after sunset. This is due to the spadefoots tendency to stay in burrows beneath the ground and only emerge at night and/or after significant rainfall (Bragg 1944, 1965).

Ponds in the surveyed area were first examined for the presence of tadpoles. If tadpoles were present a sample was taken, and a dichotomous key (Stebbins 1985) was used to identify the species. The specific feature used to determine if the tadpole was a plains spadefoot toad (*Scaphiopus bombifrons*) was the presence of oral papillae around the entire margin of the mouth. One tadpole was also kept and raised to the juvenile stage at which time it was determined to be a plains spadefoot toad by the presence of a raised bony boss between the eyes and a vertical pupil. The only other species of tadpole encountered in ponds in the area was the Western Chorus Frog (*Pseudacris triseriata triseriata),* which are easily distinguishable from spadefoot toads.

Sampling was carried out by scooping up tadpoles with a net at regularly spaced intervals of about 2 meters along the edge of the pond until a sample size was reached that was deemed sufficient for that particular pond. Sufficient sample size was based on the size of the pond, and the estimated density of tadpoles present. In all cases an attempt was made to sample no more than approximately 20% of the individuals in a given pond, in order to minimize the impact of on sampling recruitment. The number of individuals sampled per pond ranged from 11 (pond 2) to 50 (ponds 4 and 8). Because the water was so turbid often the number of tadpoles present was estimated based on movement within the water and the frequency of tadpoles surfacing to gulp air. Thus the estimates of the number of tadpoles present was very approximate and was used only as a rough guide to determine the number of samples to be taken.

Tadpole sizes tended to be similar within individual ponds, but were often quite different between ponds. Snout-vent lengths (SVL) for sampled individuals ranged from less than 1 cm to greater than 2.5 cm. Once the tadpoles had been caught they were placed into 1.5 ml micro-centrifuge tubes containing 95% ethanol for preservation. Either the whole animal or just the tail and a portion of the back musculature were preserved depending on the size of the animal. This was necessary because tadpoles in many of the ponds were approximately 20-30 days old and were too large to fit into the micro-centrifuge tubes. This situation arose because breeding ponds were not located until well after the actual breeding event took place, and many of the tadpoles had already grown to sizes reaching 2.5 cm (SVL).

Multiple GPS readings were also taken at each of the ponds using a Magellan GPS Pioneer hand-held GPS unit. Readings were taken at each pond at least twice, and an effort was made to take these readings at different times of the day. This was done because the number of satellites that the GPS unit could establish contact with varied depending on the location of the satellites when the readings were taken. Also, it was indicated by the manufacturer that a certain amount of introduced error was present in location readings. To try to minimize the impact of this introduced error, several readings were taken and then an average was calculated. These location readings were later used to calculate inter-pond distances, which were then used in the data analysis to examine isolation by distance effects (Table 2).

Laboratory Methods

All laboratory work was done using protocols developed by Dr. Colin Hughes and his associates for finding and developing microsatellite loci. The first step in developing

microsatellite primers was to locate microsatellite sequences within the spadefoot genome. This was done by creating a DNA library, which was then screened in order to isolate fragments of DNA that contained microsatellite sequences. The specific microsatellite sequence that was probed for was the trinucleotide repeat AAT. This trinucleotide was chosen because it had been detected successfully in the past, and seemed more common than other trinucleotides that had been tried (C. Hughes, personal communication).

Genomic DNA was isolated from muscle tissue taken from a single adult spadefoot toad using a phenol-chloroform extraction. Once genomic DNA had been isolated, it was then digested with the restriction enzyme Dpn II and run out on a 2% agarose gel in order to separate the DNA fragments by size. The portion of the gel containing fragments of approximately 200-600 base pairs in length was cut out, and the DNA fragments were extracted from the gel and resuspended.

These size-selected fragments were then ligated to phage DNA using Stratagene's Lambda Zap Express kit. The phage DNA/toad DNA construct was then packaged into the whole phage in order to produce a complete lambda phage, which acted as a vector and was used to infect *E. coli* bacteria during the library screening process. Once the phage DNA/toad DNA constructs were packaged into the lambda phage, the phage suspension was then mixed with a suspension of XL1 Blue *E. coli* bacteria and plated out onto 150 mm LB-agar plates. These plates were incubated at 37° C for 12 hours to allow the phage to infect the bacteria and were then placed in a refrigerator. This resulted in plates containing an opaque bacterial 'lawn' that was covered with small, circular clear areas representing colonies of lambda phage.

The DNA library was then screened in order to identify phage colonies, which were comprised of clones that contained toad DNA inserts with microsatellite sequences. Nylon membranes (Immobilon) were laid on top of the agar plates containing the colonies, removed, and treated in order to bind the clone DNA to the membrane. These membranes were then probed by adding an oligonucleotide probe end-labeled with radioactive phosphate ($\alpha\ ^{32}P$). The probe is a DNA sequence complementary to the sequence of interest. For example, to screen for the trinucleotide repeat AAT, a probe would have the sequence TTA repeated ten times resulting in an oligonucleotide that is 30 base pairs long. This length helps to insure probe specificity (i.e. the probe will only bind to complementary sequences) and reduce the number of false positives that occur during the screening process (C. Hughes, personal communication).

After the probe was hybridized with the filters, the filters were washed and placed on Kodak XAR film for exposure. Primary positive clones, clones that contained the sequence of interest (AAT) and had bound the radioactive oligonucleotide probe, appeared as dark spots on the film. The films were then aligned with the clones on the agar plates so that the dark spots on the film matched up with their clone of origin on the agar plates. These primary positive clones were then recovered from the agar plates using a transfer pipette, and resuspended in a solution of suspension medium and chloroform. The suspension was then re-plated at a lower density and re-probed in the same manner as described above, to produce secondary positive clones. This step was repeated a third time to produce the tertiary positive clones, which were then sequenced in order to determine the actual sequence of the putative microsatellite. This series of screenings is important because it ensures that after several repetitions of probing and re-

plating at a lower density, each clone contains only a single toad DNA insert, and removes false positives before the sequencing step.

Before sequencing, the cloned toad DNA was excised from the phage DNA to form a plasmid containing the toad DNA insert and a bit of the surrounding phage DNA. This was done because the plasmids are easier to work with than the whole lambda phage with the DNA packaged inside. After the plasmids were generated, they were digested using the restriction enzymes Pst I and EcoR1 and run out on a 2% agarose gel. A Southern blot was then made of the gel by first denaturing the DNA using a solution of 1.5 M NaCl and 0.5 N NaOH, and then transferring the DNA to a nylon membrane (Immobilon). The DNA was bound to the membrane by baking at 80° C for approximately 30 minutes and then by UV crosslinking using a UV Stratalinker (Stratagene). The membrane was then probed using the same $\alpha^{32}P$ labeled oligonucleotide probe that was used in the initial screening of the DNA library. The probed membrane was then placed on x-ray film in order to identify clones that successfully bound the probe. The clones that bound the probe were then selected for sequencing, while the clones that failed to bind the probe were then discarded as false positives.

A total of 33 clones were found to be strongly positive, as determined by the Southern blot, and were sequenced according to the protocol cited above. Two different sequencing primers, PBKCMV-1 and PBKCMV-2, were used during sequencing. These primers were designed to each recognize a specific sequence on opposite sides of the cloned plasmid. This can be visualized in the following manner; the plasmid is a short, circular piece of DNA, which is cut in the middle by a restriction enzyme and into which

the cloned toad DNA is inserted. The two primers each recognize a specific sequence of nucleotides near the edge of the plasmid close to the DNA insert and bind to that region. Each of these primers then initiates sequencing through the short segment of plasmid DNA and into the DNA insert. By sequencing from either direction a segment of inserted DNA of approximately 600-700 base pairs can be sequenced, usually with some area of overlap. The transition area between the plasmid sequence and the DNA insert sequence is recognized by the presence of the sequence GATC, thus the rest of the sequence following this motif is that of the cloned DNA insert.

The radioactive phosphate isotope $\alpha^{33}P$ was also added to the sequencing reaction so that the products could later be viewed using autoradiography. The reactions were placed in a Omnigene thermocycler (Hybaid) which ran the following program: 15 seconds at 95° C, 15 seconds at 55° C, and 30 seconds at 72° C; this program was then repeated 30 times. The products of the sequencing reactions were loaded on a denaturing acrylamide gel and run out for 7000 volt hours or 5000 volts hours for the PBKCMV-1 and PBKCMV-2 primer products respectively. The gels were then dried and placed on x-ray film for exposure. The DNA sequence was then read directly from the film, and clones with microsatellite sequences were identified.

Only clones that contained uninterrupted AAT trinucleotide repeats of eight repeats or more in length were considered as candidates for PCR primer development. This minimum microsatellite repeat length was established because of the correlation between repeat length and polymorphism for a given locus. Specifically, microsatellites with eight or more repeats tend to be more polymorphic than those with less than eight repeats (C. Hughes, personal communication).

The entire readable sequence of clones containing suitable microsatellite loci was then entered into the program Oligo 4.04 (National Biosciences Inc.) and used to design PCR primers. Several criteria were used to determine which sequences in the flanking region of the microsatellite would be best suited for use as PCR primers. The first of these was melting temperature within each primer sequence. Melting temperature is the temperature above which binding between two DNA sequences becomes unstable and the two pieces of DNA separate. Primers were designed so that the 3' end had a higher melting temperature than the 5' end of the primer. This is because if the 3' end of the primer is not fully bound to the template DNA, then DNA replication cannot proceed and no PCR products will be produced. This ensures that only primers that are fully bound will amplify any products during PCR. If the 3' end of the primer has a lower melting temperature than the 5' end, then it becomes more likely that non-specific binding will occur and portions of the sample DNA other than the microsatellite region will be amplified. This often results in 'miscellaneous' PCR products being produced and can make scoring gels more difficult, due to the presence of extraneous bands and lighter colored bands than the microsatellite loci that was intended to be amplified.

Upper and lower primer sequences also had to be chosen so that no heteroduplexes would form between the two primers. This occurs when there are complementary sequences within the primers that cause the two primers to bind together at temperatures at or near the predicted annealing temperature. Heteroduplex formation effectively reduces the concentration of functional primers in a reaction because primers that have formed heteroduplexes are no longer able to bind to the template DNA. Homoduplexes represent a similar situation and occur when there are complementary

sequences within a single primer, causing the primer sequence to bend back and 'stick' to itself. This can also inhibit binding to the template DNA and sequences that contained stable homoduplexes were also rejected as possible primer sequences.

The melting temperatures of the upper and lower primers also had to be kept as close together as possible. This is because melting temperature is directly related to annealing temperature, or the temperature at which the primers will bind to the template DNA. Because both the upper and lower primers must function in the same PCR reaction, they must have melting temperatures that are similar enough to allow both to function at the single annealing temperature that will be used during the PCR reaction. If annealing temperatures are too dissimilar, one primer may have non-specific binding at unwanted areas in the template DNA while the other primer may not bind at all. However, when melting temperatures are kept relatively close PCR conditions can be optimized so that both primers will bind only to the flanking region on either side of the microsatellite, and only that area will be amplified.

Eight pairs of oligonucleotide PCR primers, with lengths ranging from 18 to 22 base pairs, were designed and ordered from Integrated DNA Technologies. PCR conditions were then optimized for each of these primer pairs. The optimization procedure consisted of testing various melting temperatures and $MgCl_2$ concentrations for each primer pair. All primer pairs used annealing temperatures (T_A) of either 50° C or 55° C, and none required the addition of $MgCl_2$. Once primer pairs had been optimized they were used in PCR to generate genotypes at each of five microsatellite loci for each of the sampled individuals. The other three loci were dropped due to low levels of polymorphism.

PCR reactions were set up according to the protocols described in Hughes (1996). The nucleotide dATP labeled with the radioactive isotope α ^{35}S was added to the reaction so that the products could later be viewed using autoradiography. Reactions were then placed in a thermocycler (Hybaid); reaction temperatures and temperature duration were controlled using a PCR program of the following format:

-93° C for 90 seconds, repeated one time.
-93° C for 30 seconds, then annealing temperature (T_A) for 30 seconds, then 73° C for 30 seconds, repeated 40 times.
-73° C for 90 seconds repeated one time.

The PCR products, along with a M13 PCR size marker, were loaded onto a 7% acrylamide, 7M urea, denaturing gel and run out for different durations depending on the size of the products. The gels were then dried and placed on Kodak Biomax film for exposure. Exposure times varied from gel to gel depending on the amount of α ^{35}S incorporated in the PCR products. The films were then developed, and genotypes were scored by using the M13 size marker to determine the overall length of the PCR products. Products with different overall lengths were considered to be individual alleles at a given locus. This procedure was repeated for all 305 individuals at each of the 5 microsatellite loci.

Statistical Analyses

GENEPOP version 3.1d (Raymond and Rousset 1995) was used to perform all of the following tests except for the Analysis of Molecular Variance (AMOVA) and Multidimensional Scaling (MDS) which were performed using Arlequin version 2.0

(Schneider et al 2000) and STATISTICA '99 (StatSoft, Inc. 1999) respectively. All of the information given below regarding the details of the tests performed was taken from the GENEPOP version 3.1d user's manual (Raymond and Rousset 1995), the Arlequin version 2.0 users manual (Schneider et al. 2000), and StatSoft's Electronic Statistics Textbook (StatSoft, Inc. 1997).

Basic information such as allele frequencies, observed and expected genotype frequencies, observed and expected number of homozygotes and heterozygotes, and estimates of F_{is} were calculated for each locus in each population using GENEPOP. A genotypic matrix and a table of allele frequencies for each locus and for each population were also created.

Deviations from Hardy-Weinberg equilibrium were tested for at each locus in each population, by testing the null hypothesis (H_o) of random union of gametes, and the specific alternative hypothesis of heterozygotes deficiency. The exact P-value of this test was estimated using a Markov chain method according to the method outlined in Guo and Thompson (1992). A global test for heterozygote deficiency, in which results were averaged across populations and then across loci, was also performed.

Linkage disequilibrium was tested for in a similar manner by examining the null hypothesis (H_o) that genotypes at one locus are independent from genotypes at the other locus. GENEPOP was used to first create contingency tables for all pairs of loci in each population, and then perform a probability test for each table using a Markov chain. A global test (Fischer's method) for each pair of loci was also performed across all populations.

The presence of null alleles in the data set was suspected because significant heterozygote deficiencies were observed at four of the five loci. Chakraborty's method of estimating the frequency of null alleles from apparent heterozygote deficiencies (Chakraborty et al. 1992) was used to estimate the frequency of null alleles present in the data set. This was done for all 9 ponds at all loci except SbAAT 8 where no heterozygote deficiency was observed.

Once the frequency of null alleles had been calculated, the estimated frequency of the null allele was subtracted from one, which gave the total frequency of all of the observable alleles (the frequency of the null allele and all visible alleles should sum to one). This value was then partitioned according to the original proportions of the observable alleles to give the adjusted allele frequencies for each visible allele. These allele frequencies were then used to calculate new Hardy-Weinberg expected heterozygote frequencies ($2p_{ij}q_{ij}$). The corrected expected heterozygote frequencies were then compared to the observed heterozygote frequencies in order to determine if the null allele correction corrected the observed heterozygote deficiency.

Measures of genetic distance based on allele frequency (F-statistics) and allele length (Rho-statistics) were calculated using GENEPOP. F_{is}, F_{it} and F_{st} were calculated across all populations for each locus using a weighted analysis of variance (Cockerham 1973; Weir and Cockerham 1984). The analogous measures of correlation in allele size (rho-st, see Rousset, 1996) were estimated using the same technique (Michalakis and Excoffier 1996).

Pairwise estimates of genetic distance based on allele frequency (F_{st}) and allele length (R_{st}) were also calculated using GENEPOP. These were calculated for each pair

of populations at each locus and also for each pair of populations across all loci. Multi-locus estimates were computed by GENEPOP following the method outlined by Weir and Cockerham (1984), and were later used to test for isolation by distance.

Population differentiation was tested for all populations, and all pairs of populations by using the distribution of alleles at each locus to test the null hypothesis (H_o) that the allelic distribution is identical across populations. This was done in GENEPOP by creating contingency tables for each locus containing the frequency of each allele at that locus in each population. Then an unbiased estimate of the P-value of the probability test was obtained using a Markov chain method as described in Raymond and Rousset (1995). This same method was used for examining population differentiation between all population pairs, but in this case the test was performed for all pairs of populations at each locus and also across all loci.

All significance tests in which P-values were calculated for multiple comparisons, including linkage disequilibrium, Hardy-Weinberg equilibrium, and genetic differentiation were adjusted using the sequential Bonferroni correction as described by Rice (1989). Because the genetic data was non-parametric, data re-sampling methods were used in order to generate estimates of p-values for all calculated statistics.

After the presence of genetic differentiation between ponds had been tested as described above, the next step was to test for the presence of specific patterns of genetic variation. Isolation by distance was tested using a Mantel's test and patterns of differentiation between specific groups of ponds was tested for by using an Analysis of Molecular Variance (AMOVA). Multi-Dimensional Scaling was used to examine graphically the pattern of pairwise differentiation among populations.

The multi-locus, pairwise estimates of F_{st} and R_{st} were put into matrix form and were then used along with a matrix of pairwise geographic distances to do an analysis of isolation by distance. GENEPOP used a Mantel's test (Mantel 1967) to test significance of the regressions of geographic distance and F_{st} or R_{st}. These tests were performed on both transformed genetic (F_{st} /1- F_{st}) and spatial distances (natural log of distance), as well as the untransformed genetic and spatial distances.

An Analysis of Molecular Variance (AMOVA, Excoffier et al. 1992) was performed using Arlequin version 2.0 (Schneider et al 2000) to examine hierarchical population genetic structure. Two different groupings of ponds were used, in the first analysis all ponds were put into a single group and among population and within population variance components were calculated. In the second analysis ponds were put into 3 different groups based on their geographic locations. Group 1 contained ponds 1 and 2, group 2 contained ponds 3, 4, 5 and 6, and group 3 contained ponds 7, 8, and 9. For this analysis the following variance components were calculated: among groups, among populations within groups, and within populations. Estimates of the fixation indices (F_{st} or R_{st}) were also calculated for each analysis. The significance of the fixation indices was tested using a non-parametric permutation approach that is described in Excoffier et al. (1992).

Multi-dimensional scaling was performed using STATISTICA '99 (StatSoft, Inc., 1999) to look for patterns in pairwise genetic distances between ponds. The input data for this analysis consisted of square matrices made up of multi-locus, pairwise genetic distances for all pairs of ponds. Separate matrices were constructed for pairwise F_{st} and R_{st} values. The data was analyzed using three dimensions and stress values were

generated to evaluate goodness of fit. The data was then plotted both in three-dimensions and also in three separate two-dimensional plots, one for each pair of dimensions.

CHAPTER III

RESULTS

A total of 304 individuals were genotyped at five microsatellite loci using the first five of the primer pairs listed in Table 1. This produced scorable genotypes for 282 of the 304 individuals. The number of loci that produced usable genotypes ranged from 1 to 5 per individual.

Microsatellite and primer structure, number of alleles, expected heterozygosity for each locus and annealing temperatures (T_A) are shown in Table 1. Three of the five microsatellite loci were relatively polymorphic, having either 10 or 12 alleles; the other two loci were substantially less polymorphic, having only 3 and 4 alleles respectively.

Most alleles varied in size by multiples of three as would be expected under a stepwise mutation model. However, there were exceptions to this pattern in which alleles varied by only a single base. This seems likely to be caused by mutations in the region flanking the microsatellite rather than mutations in the microsatellite itself, as all of the microsatellites were made up of uninterrupted repeats of AAT and would be expected to mutate in whole repeat units (3 bp) (Eisen 1999).

The distribution of allele sizes across all ponds for each locus is given in Table 2. Most allele sizes tended to fall in the shorter end of the size range distribution, with the exception of locus SbAAT 91 where the longest allele (101 bp) was the most common.

Allele frequencies at each locus for all nine ponds are also shown. None of the allele frequencies shown have been corrected for null alleles.

The observed and expected heterozygosities and sample size for each locus are shown in Table 4. The results of the test for deviations from Hardy-Weinberg equilibrium showed that all loci except SbAAT 8 had a significant deficiency of heterozygotes for most ponds when compared to Hardy-Weinberg expected genotype frequencies. The cause of this deficiency could be due to the presence of null alleles, or perhaps heterozygosity is being lost at the examined loci due to the effects of inbreeding, founder events, or genetic drift. These possible explanations for the intriguing paucity observed in heterozygosity for 80% of the loci used in this study will be discussed in more detail later on.

The results of the test for linkage disequilibrium for each pair of loci are presented in Table 6. Out of the ten comparisons, only one P-value was significant after the sequential Bonferroni correction was applied.

The exact tests for genic differentiation based on allele frequency showed that all possible pairs of ponds were significantly different (Table 8). The initial P-values were then corrected by applying the sequential Bonferroni correction (Rice 1989). After correction all pairs of ponds were still significantly different.

Estimates of Fst and Rst (Table 4) were relatively high for most loci. Values of Fst ranged from 0.056 for locus SbAAT 91 to 0.115 for SbAAT 28, and values of Rst ranged from 0.040 for SbAAT 91 to 0.163 for SbAAT 28. The pairwise estimates of Fst and Rst (Table 7) ranged from 0.004 to 0.227 for Fst, and 0.000 to 0.324 for Rst.

In addition to pairwise estimates for Fst and Rst, these estimates were plotted along with interpond distances (Figures 2 and 3) and tested for the presence of isolation by distance using Mantel's test. Although the plotted data appeared to indicate isolation by distance, the results of the Mantel's test showed that the null hypothesis of no isolation by distance could not be rejected for Fst or Rst (P=0.129 and P=0.345 respectively).

The results of the multi-dimensional scaling plots showed no discernible clustering of ponds with regard to genetic distance. The output from this test was plotted in three dimensions and also in two dimensions using all possible combinations of the three dimensions from the three-dimensional plot. The amount of stress for the Fst (stress = 0.023) and Rst (stress = 0.007) multi-dimensional scaling plots was low indicating a relatively good fit of the plot to the data.

Table 1. Microsatellite and primer structure by locus.

Locus	Microsatellite structure	No. of alleles	Expected He	Primer Sequence (5' - 3')	TA (° C)
8	$(AAT)_9$	12	0.703	GTGGCAGGGACATACAGT CCAGCATACACTAAGCAACTC	50
28	$(AAT)_{11}$	3	0.477	GGGCAACTTTAGCGTCTT AACTGTTGGCGCTATATAAAT	50
49	$(AAT)_8$	10	0.692	TTGGCTCTGACTACTTGTTG CAGTCTCTCCCTACCTTAAAT	50
62	$(AAT)_{15}$	12	0.769	CCAAACTGGCAGTATTCAGA TGTTGGTGCCGTGTGTTA	50
91	$(AAT)_{10}$	4	0.615	CATTAAAGCTCGTAATAAT AGGTGCTGTAAATACTCA	50
15	$(AAT)_{12}$	---	---	ATAAATCCTGGATCTTTCTC GGGAAGTAGATTAAATTATTG	55*
73	$(AAT)_9$	---	---	CTGGGATCGTCTTCCAAT GATGCCCTTCAACTACAATG	55*
117	$(AAT)_9$	---	---	GGGCCATATTATTTTAGGAA TGGCGCTATATAAATAAAAGAT	55*

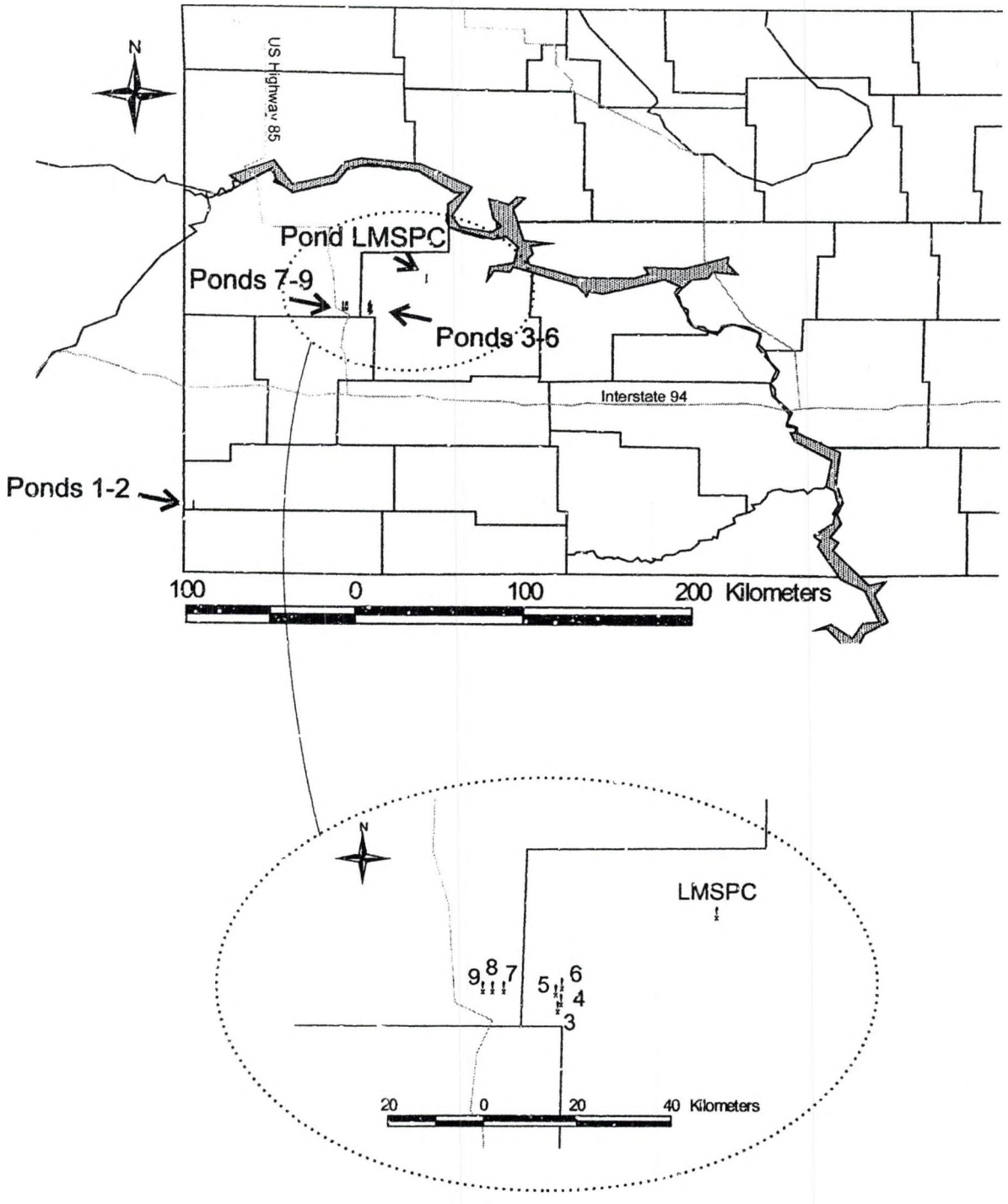

Figure 1. Sampling locations in western North Dakota.

Table 2. GPS readings of pond locations.

Pond #	UTM N	UTM E
Pond #1	5129540	13 579508
Pond #1	5129550	13 579464
Pond #1	5129551	13 579530
(ave.)	5129547	13 579500
Pond #2	5129128	13 579529
Pond #2	5129143	13 579618
(ave.)	5129136	13 579573
Pond #3	5248810	13 648435
Pond #3	5248747	13 648433
Pond #3	5248846	13 648468
(ave.)	5248801	13 648445
Pond #4	5249441	13 648690
Pond #4	5249442	13 648689
(ave.)	5249442	13 648689
Pond #5	5250875	13 647615
Pond #5	5250771	13 647645
Pond #5	5250793	13 647647
(ave.)	5250813	13 647635
Pond #6	5251517	13 648212
Pond #6	5251540	13 648275
(ave.)	5251529	13 648243
Pond #7	5251374	13 639335
Pond #7	5251355	13 639445
(ave.)	5251365	13 639390
Pond #8	5251310	13 638432
Pond #8	5251332	13 638447
Pond #8	5251338	13 638483
(ave.)	5251327	13 638454
Pond #9	5251340	13 637565
Pond #9	5251346	13 637583
Pond #9	5251267	13 637578
(ave.)	5251318	13 637575

Table 3. Microsatellite allele frequencies by locus and pond.

Locus:	SbAAT 8										
	Alleles in bp										
	165	171	174	176	177	178	179	180	188	189	195
Pond											
1	0.214	0	0.214	0	0.45	0	0.095	0	0	0.02	0
2	0.071	0.07	0	0	0.43	0	0.214	0	0	0.07	0.143
3	--	--	--	--	--	--	--	--	--	--	--
4	0.182	0	0.159	0.545	0	0.023	0	0	0.023	0.07	0
5	0.488	0.01	0.275	0.05	0.06	0	0.025	0	0	0.09	0
6	0.325	0	0.4	0.05	0	0	0	0	0	0.23	0
7	0.288	0	0.394	0.03	0.17	0	0	0.045	0	0.08	0
8	0.28	0	0.38	0.02	0.22	0	0	0.02	0	0.08	0
9	--	--	--	--	--	--	--	--	--	--	--
overall allele frequencies	0.264	0.01	0.26	0.099	0.19	0.003	0.048	0.009	0.003	0.09	0.02

Locus:	SbAAT 28		
	Alleles in bp		
	90	96	99
Pond			
1	--	--	--
2	0.188	0.81	0
3	0.32	0.68	0
4	0.196	0.57	0.239
5	0.571	0.39	0.036
6	0.235	0.53	0.235
7	0.652	0.35	0
8	0.63	0.37	0
9	0.225	0.78	0
overall allele frequencies	0.377125	0.56	0.064

Table 3 cont. Microsatellite allele frequencies by locus and pond.

Locus:	SbAAT 49									
	Alleles in bp									
	110	113	119	125	130	134	136	137	142	145
Pond										
1	0	0.17	0.452	0.214	0	0	0.167	0	0	0
2	0	0.13	0.688	0.063	0	0	0.125	0	0	0
3	0	0.32	0.463	0.056	0	0	0.167	0	0	0
4	0	0.51	0.316	0.026	0	0	0.105	0.039	0	0
5	0	0.48	0.283	0.067	0.03	0.033	0.083	0	0.017	0
6	0	0.79	0.147	0	0	0	0.029	0	0	0.03
7	0	0.28	0.175	0.075	0	0	0.375	0	0	0.1
8	0.023	0.21	0.364	0.136	0	0	0.273	0	0	0
9	0	0.69	0.292	0.021	0	0	0	0	0	0
overall allele frequencies	0.003	0.4	0.353	0.073	0	0.004	0.147	0.004	0.002	0.01

Locus:	SbAAT 62											
	Alleles in bp											
	108	109	114	123	129	132	135	136	138	141	144	147
Pond												
1	0	0	0.219	0.5	0	0	0	0	0.219	0	0	0.063
2	0.643	0	0.214	0.071	0	0	0	0	0.071	0	0	0
3	0.208	0	0.083	0.708	0	0	0	0	0	0	0	0
4	0	0	0.327	0.365	0	0	0	0	0.115	0.02	0.173	0
5	0.167	0	0.077	0.321	0.08	0	0.064	0	0.295	0	0	0
6	0	0.06	0.265	0.303	0	0	0.265	0	0.059	0	0	0
7	0	0	0.214	0.381	0	0	0.071	0.024	0.31	0	0	0
8	0.304	0	0.109	0.326	0.07	0.043	0	0	0.152	0	0	0
9	0.467	0	0	0.167	0	0	0	0.1	0.267	0	0	0
overall allele frequencies	0.198777778	0.01	0.168	0.355	0.02	0.005	0.044	0.014	0.165	0	0.019	0.007

Table 3 con't. Microsatellite allele frequencies by locus and pond.

Locus:	SbAAT 91			
	Alleles in bp			
	93	95	96	101
Pond				
1	0.412	0.06	0	0.529
2	0.2	0.55	0	0.25
3	0.239	0.26	0	0.5
4	0.75	0.17	0	0.083
5	0.179	0.39	0.054	0.375
6	0.455	0.09	0	0.455
z	0.25	0.13	0.125	0.5
8	0.292	0.25	0.083	0.375
9	-	-	-	-
overall allele frequencies	0.347125	0.24	0.033	0.383

- : denotes a lack of scorable genotype data for all loci at a at a given pond (dropped from analysis).
0 : denotes a lack of scorable genotype data for a specific locus at a at a given pond.

Table 4. Observed and expected heterozygosities, F_{st}, and R_{st} for each locus.

Locus		Pond1	Pond 2	Pond 3	Pond 4	Pond 5	Pond 6	Pond 7	Pond 8	Pond 9
SbAAT-8										
n		21	7	20	22	41	20	33	25	0
Ho		0.619	0.857	0.600	0.455	0.610	0.650	0.697	0.640	---
He		0.711	0.791	0.610	0.653	0.688	0.699	0.737	0.736	---
Fst	0.104									
Rst	0.049									
SbAAT-28										
n		0	8	25	23	28	17	23	23	20
Ho		---	0.375	0.400	0.261	0.143	0.000	0.348	0.217	0.050
He		---	0.325	0.444	0.598	0.527	0.627	0.464	0.476	0.358
Fst	0.115									
Rst	0.163									
SbAAT-49										
n		21	8	27	37	30	17	20	22	24
Ho		0.190	0.125	0.185	0.459	0.200	0.118	0.150	0.273	0.333
He		0.711	0.525	0.668	0.634	0.684	0.357	0.756	0.749	0.451
Fst	0.085									
Rst	0.137									
SbAAT-62										
n		16	7	24	26	39	17	21	23	15
Ho		0.188	0.286	0.167	0.308	0.462	0.059	0.286	0.348	0.333
He		0.671	0.571	0.457	0.730	0.777	0.750	0.725	0.777	0.697
Fst	0.093									
Rst	0.076									
SbAAT-91										
n		17	10	23	12	28	11	32	24	0
Ho		0.235	0.100	0.130	0.000	0.179	0.000	0.188	0.167	---
He		0.563	0.626	0.639	0.420	0.682	0.606	0.667	0.720	---
Fst	0.056									
Rst	0.040									

Table 5. Significance for deviations from H-W equilibrium, P-values have been corrected using the sequential Bonferroni technique (Rice 1989).

Locus 8			
Population Pair	p-value	signif (0=no, 1=yes)	
1	0.2259	0	
2	0.8735	0	
3	0.4563	0	
4	0.0069	0	
5	0.1781	0	
6	0.3253	0	
7	0.4056	0	
8	0.1977	0	
9	-		
		0	# of signif. results

Locus 28			
Pond	p-value	signif (0=no, 1=yes)	
1	-		
2	1	0	
3	0.4722	0	
4	0.0002	1	
5	0	1	
6	0	1	
7	0.2171	0	
8	0.0121	1	
9	0.0006	1	
		5	# of signif. results

Locus 49			
Pond	p-value	signif (0=no, 1=yes)	
1	0	1	
2	0.0051	1	
3	0	1	
4	0.0145	1	
5	0	1	
6	0.0015	1	
7	0	1	
8	0	1	
9	0.0603	0	
		8	# of signif. results

Table 5 con't. Significance for deviations from H-W equilibrium, P-values have been corrected using the sequential Bonferroni technique (Rice 1989).

Locus 62

Pond	p-value	signif (0=no, 1=yes)	
1	0	1	
2	0.0629	0	
3	0.0337	0	
4	0	1	
5	0	1	
6	0	1	
7	0.0005	1	
8	0.0002	1	
9	0.0057	1	
		7	# of signif. results

Locus 91

Pond	p-value	signif (0=no, 1=yes)	
1	0.0016	1	
2	0	1	
3	0	1	
4	0.0003	1	
5	0	1	
6	0.0001	1	
7	0	1	
8	0	1	
9	-	0	
		8	# of signif. results

Table 6. Results of test for linkage disequilibrium, P-values were evaluated using the sequential Bonferroni technique (Rice 1989). Significant P-values are denoted with an asterisk (*).

Locus Pair	Chi 2	df	p-value
sb8 & sb28	11.23	14	0.66788
sb8 & sb49	24.103	16	0.08727
sb8 & sb62	25.142	16	0.06736
sb8 & sb91	23.163	16	0.10946
sb28 & sb49	39.799	16	0.000830*
sb28 & sb62	25.295	16	0.06479
sb28 & sb91	23.016	14	0.06091
sb49 & sb62	31.529	18	0.02498
sb49 & sb91	20.834	16	0.18499
sb62 & sb91	15.958	16	0.45592

CHAPTER IV

DISCUSSION

Allele length and distribution

The number of alleles observed at each locus in this study ranged from a low of 3 for locus 28 to a high of 11 for locus 8. Allele lengths ranged from 90 bp for locus 28 to 189 bp for locus 8. The number of alleles observed at most loci examined in this study are higher than those reported for microsatellites in several other species of amphibians. Rowe et al. (1999) reported finding an average of 1.5 to 2.8 alleles per locus in populations of *Bufo calamita*, and Scribner *et al.* (1994) observed 6 alleles in the one microsatellite locus examined in *Bufo bufo*. In comparison, *S. bombifrons* loci 8 and 62 have 11 and 10 alleles respectively giving a level of polymorphism that is somewhat higher than most other amphibian microsatellite loci that have been reported. The remaining loci have levels of polymorphism that more closely resemble those that have been previously reported.

Allele lengths reported in several previous amphibian studies that utilized microsatellites, fell between 109 bp and 266 bp (Goebel *et al.* 1999).
The allele lengths observed in this study fall in this range with the exception of alleles at loci 28 and 91, which ranged between 90 and 101 bp. This difference however does not seem substantial and most alleles follow the size pattern previously observed.

The data did show some correlation between allele length and polymorphism with loci having shorter alleles generally also being less polymorphic. Both of the most polymorphic loci (loci 8 and 62) had alleles that ranged between 108 and 189 bp, while the least polymorphic (loci 28 and 91) had alleles that were 101 bp long or less. This pattern has been previously documented in microsatellites in many other organisms ranging from yeast to humans (Jin *et al.* 1996 and Weirdl *et al.*1997), and is expected under the slip strand mispairing model of microsatellite mutation.

Hardy-Weinberg Equilibrium and Linkage Disequilibrium

The test for linkage disequilibrium among the five loci revealed significant linkage for only one out of the ten comparisons (loci 28 & 49) after sequential Bonferroni correction. This indicates that overall the loci are independent of each other and data generated from each can be treated as independent observations.

The exact test for deviations between the expected and observed values for heterozygosity under Hardy-Weinberg equilibrium showed significant heterozygote deficiencies for all loci except locus 8. The possible explanations for these deficiencies can be grouped into 2 categories: population level processes (Wahlund effect and inbreeding) and locus specific processes (null alleles, short allele dominance, and selection).

Population level processes such as the Wahlund effect and inbreeding have been observed to produce heterozygote deficiencies in a number of species (Gibbs et al. 1998 and Green et al. 1996). The life history of spadefoots, including a fairly low dispersal capability and a tendency towards philopatry, would at first seem to suggest that either of these explanations would be a good fit for this system. However, although the Wahlund

effect and inbreeding could both cause a reduction in observed heterozygosities below the level expected under Hardy-Weinberg, both of these processes should affect all loci equally. This is not the case in this study as locus 8 showed no heterozygote deficiency and fit well with Hardy-Weinberg expectations. This inconsistency of heterozygote deficiency across loci would seem to indicate that a locus specific process may be playing a role in maintaining heterozygosity at locus 8.

The first two locus specific processes that will be examined here are null alleles and short allele dominance. These two effects will be considered together because they are both artifacts of the PCR and allele scoring process that generated the raw genotype data that was compared against Hardy-Weinberg expectations.

Null alleles occur when certain alleles are not amplified during the PCR process due to the occurrence of mutations in the DNA sequence flanking the microsatellite where the PCR primers bind (Callen *et al.* 1993; Paetkau and Strobeck 1995). This lack of amplification for one of the two alleles present in a heterozygote would lead to only one band being observed on a scoring gel instead of two. This individual would then be mis-scored as a homozygote. If this were to occur for several or more individuals at a given locus this process could produce an apparent deficiency of heterozygotes for the locus.

Short allele dominance occurs in heterozygotes where one short allele and one long allele are present. In this case it has been observed that the short allele will be preferentially amplified over the long allele, sometimes causing the non-detection of the long allele (Wattier *et al.* 1998). This effect has been shown to increase with increasing difference in size between the short and long alleles (Wattier *et al.* 1998). The molecular

details of the process have not been fully elucidated, but it has been shown that certain parameters in the PCR process such as extension time and the concentration of *Taq* polymerase can affect the frequency in which short allele dominance occurs.

Although either of these locus specific effects could cause the heterozygote deficiencies observed in this study, there are factors that make either of these explanations questionable. When the Brookfield method for estimating the frequency of null alleles was applied to the heterozygosity data it gave estimates of null alleles ranging from 0.05 for locus 8 to 0.66 for locus 91. The null allele estimates for the loci that were strongly deviated from Hardy-Weinberg expectations (loci 28, 49, 62, and 91) ranged between 0.36 and 0.66. These null allele estimates are not only extremely high, but these high estimates of null allele frequency appear for 4 out of 5 loci. It seems improbable that null alleles would occur in such high frequency at 80% of the loci examined. Similar explanations have also been rejected by other authors in similar cases (Gibbs et al. 1998).

The same logic can be applied with regard to short allele dominance being responsible for the observed deficiency of heterozygotes. It seems reasonable that short allele dominance could account for heterozygote deficiency at one or even two loci but it seems unlikely to be occurring at 4 out of 5 loci. Also short allele dominance usually occurs in heterozygotes with one short and one long allele and the effect becomes greater as the length of the longer allele increases. Wattier et al. described this effect generally occurring when allele lengths reached 285 bp or longer. The longest observed allele in this study was allele 189 at locus 8. The observed allele lengths would again suggest that short allele dominance should not play a large role in generating the heterozygote deficiencies observed in this study.

If none of the above mentioned processes are generating heterozygote deficiency in this system then what would be a reasonable explanation for the observed patterns? The answer may be a combination of a population level process (inbreeding) and a locus specific process (balancing selection). Instead of focusing on why 4 loci are out of Hardy-Weinberg perhaps the focus should be on explaining why locus 8 is in Hardy-Weinberg. Hardy-Weinberg equilibrium conditions may not be expected in this system at all due to aspects of the life history of the spadefoot toad such as long periods of underground inactivity, a low dispersal rate and some level of philopatry. These all suggest that a fairly high amount of inbreeding would not be unexpected in local populations, and that this inbreeding would then indeed play an important role in generating the low levels of heterozygosity observed in this system.

But if this is the case then why is the effect present at only 4 of the 5 loci examined? It is possible that locus 8 is somewhat of an anomaly and the higher levels of heterozygosity observed could be a result of linkage with a gene under balancing selection, or simply that this locus has not been purged of genetic variation because the process is not occurring at a uniform rate for all loci. Other explanations for the observed pattern are possible but seem less parsimonious because they require explaining apparent deficits in heterozygotes for four loci rather then why locus 8 is in Hardy-Weinberg.

It seems that more loci would have to be examined in this system to see if they exhibit the severe heterozygote deficiencies observed in loci 28, 49, 62 and 91. If so, this would lend more credence to the idea that loci 8 is anomalous in this system. On the other hand, if most of the new loci did not show heterozygote deficiencies then the

observed heterozygote deficiencies could perhaps be attributed to the effect of null alleles and or short allele dominance.

Patterns of Genetic Differentiation

Exact tests were performed to test for genetic differentiation between each pair of sampled breeding ponds using allele frequencies from all five loci for each pond. The results showed significant differentiation between all pairs of ponds after sequential Bonferroni correction. Significant genetic differentiation occurred between ponds separated by very small distances such as ponds 1 and 2, which were separated by a distance of only 418 meters. This result is not isolated overall, 5 of the pairwise comparisons were between ponds that were separated by less than 1000 meters. In contrast, several other studies of population genetic structure in amphibians found little or no genetic structuring at these scales (Newman and Squire 2001).

Significant genetic differentiation between all ponds also illustrates that the forces driving the genetic differentiation are occurring at a fine spatial scale throughout the system and are not only isolated to a few ponds. It also suggests that dispersal over even moderate distances is restricted because of the xeric environment that these toads favor. Without the homogenizing effects of gene flow, the genetic differences brought about by genetic drift are allowed to accumulate even between narrowly separated ponds.

After determining that significant genetic differences were present between ponds, the next task was to measure the magnitude of these differences. This was done by calculating Fst and Rst values for each locus, and across all loci for all pairs of ponds (Tables 4 and 7). The Fst values for each of the five loci averaged across all ponds ranged from 0.040 for SbAAT 91 to 0.115 for SbAAT 28. This showed that a moderate

level of genetic differentiation was present for each of the loci. The magnitude of these values is not extremely large and seems to fall in about the middle of observed Fst values in other amphibian species (Newman and Squire 2001). A similar pattern also held true for the Rst values.

Although these averaged Fst and Rst values did not suggest that strong differentiation was taking place between the sampled ponds, a somewhat different story started to emerge when individual ponds were compared with respect to genetic differentiation.

In order to determine how the genetic variation present in the system was partitioned spatially, pairwise Fst and Rst estimates were made for all pairs of ponds (Table 6). Pairwise Fst's in this study ranged from 0.004 (ponds 7 and 8) to 0.227 (ponds 2 and 6); interpond distances were 0.937 km and 140 km respectively. Previous studies of amphibian populations observed Fst's ranging from 0.014 to 0.388 at distances that are similar to those in this study (Newman and Squire 2001). Relatively high Fst values occurred for not only the most widely separated of the ponds but surprisingly also for ponds that were quite spatially close such as ponds 1 and 2 which were only 418 meters apart. These results showed that not only were the genetic differences between ponds statistically significant, but pairwise Fst's of greater than 0.20 observed for several pairs of ponds shows that the magnitude of the variation is also biologically significant (Avise 1994).

Fairly high levels of genetic differentiation at such fine scales suggests that in addition to the genetic drift some processes linked with the spadefoots life history are playing an important role in shaping genetic variation. These processes include:

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Fairly high levels of genetic differentiation at such fine scales suggests that in addition to the genetic drift some processes linked with the spadefoots life history are playing an important role in shaping genetic variation. These processes include:

extinction/recolonization, founder events, and fairly low levels of gene flow. Taken together, these life history attributes would tend to exacerbate the effects of drift and magnify its effects. The combined effects of genetic drift and secretive life history traits of the spadefoot toad serves to allow the accumulation of genetic differences, even at very fine spatial scales.

To further elucidate the overall patterns of genetic variation in this system the data was examined using AMOVA, multidimensional scaling, and a Mantel's test to check for isolation by distance.

The results of the AMOVA showed that significant genetic structure was present between all ponds ($P < 0.00001$) as measured by both Fst and Rst (Tables 9 and 10). The amount of the total variation that was found between ponds was between 6.38 and 8.05 percent for Fst and Rst respectively. The ponds were then broken up into 3 groups based on their geographic location and then tested for significant differences between these groups. The results showed that no significant structure could be detected among groups. This showed that most of the variance was contained between ponds within groups; which also suggests that these ponds, and habitat immediately surroundings them can be considered to be separate genetic groups.

In order to further elucidate the relationship between genetic differences and spatial distances separating sampling locations, the data was examined for a isolation by distance effect. The results of the test for isolation by distance showed that the null hypothesis could not be rejected for Fst or Rst ($P=0.129$ and $P=0.345$ respectively). However, when two of the outliers for Fst were dropped (ponds 3&4 and 7&9), the results became significant. There is no justification for leaving these ponds out of the

analysis. This was merely done as an exercise, because when the data were plotted the resulting graph showed a pattern of increasing Fst with increasing geographic distance as expected under an isolation by distance effect (Figures 2 and 3).

This appears to be a real, even if statistically non-significant effect, and with more sampling would likely become significant. It is also possible that the outliers fall outside of the general pattern because genetic differentiation in these ponds is not being driven by drift but rather is the result of a founder effect. This would account for the unusually high Fst's found at several of the ponds with small interpond distances. Again, the environment and the life history of the spadefoot toad lend credence to this idea. In a highly stochastic and xeric environment it would probably not be unusual for some breeding ponds to remain dry and during times of severe drought small local populations could go extinct. If this were the case the founding of new local populations by a fairly small number of founders could give rise to a large degree of differentiation even between ponds not separated by large distances.

But at distances of greater than about 1800m the observed interpond Fst and Rst values fit the isolation by distance pattern rather well as demonstrated by the significant P value obtained after dropping outliers from the analysis. Observing isolation by distance in this system is certainly not altogether unexpected given the restricted dispersal capabilities of spadefoot toads in xeric environments. If there were a great deal of long distance dispersal then isolation by distance would not be expected (Slatkin 1993), thus this observation fits well with what is known about dispersal in these amphibians.

In addition to little or no long-range dispersal, a given species must have been present in the area for a substantial period of time for isolation by distance to occur

(Slatkin 1993, Hutchinson and Templeton 1999). The area of western North Dakota that was sampled was unglaciated during the last ice age and therefore it is likely that *Scaphiopus bombifrons* has been present in the area for a long period of time.

As the different findings from the data are taken together a picture of the genetic landscape of the sampled populations begins to emerge. The system is at or near a genetic equilibrium between migration and drift as suggested by the presence of isolation by distance and geological evidence that the area has at least been suitable for spadefoot occupation for greater than about 7,000 years. In addition, significant genetic differentiation is observed at even the finest scales over which sampling took place. This taken together with the low observed heterozygosities seem to suggest that migration rates are fairly low and that drift and inbreeding are playing an important role in shaping the genetic variation in this system.

The strong genetic differentiation at fine scales coupled with the spadefoots life history also suggests that local extinction and recolonization could also be driving genetic differences at the local scale. The local populations appear to be somewhat closed systems with a low level of gene flow between them. The genetic effects of low levels of gene flow are further exacerbated by the general tendency towards philopatry in amphibians. Even though spadefoots probably cannot afford to be strictly philopatric, due to the ephemeral nature of their breeding ponds, even returning to the same areas to breed would increase the amount of inbreeding occurring in local populations because matings would not be random. If this were repeated over successive years it could certainly lead to a loss of heterozygosity as well as potentially increasing the genetic differences between local breeding aggregations assuming that founding propagules

tended to be small and were drawn from only one or a few source populations (Wade and McCauley 1988). These founder 'type' events would be in addition to true founder events that would take place if a local population went extinct and was later re-colonized. Given the relatively harsh climate and potential for high levels of environmental stochasticity it does not seem unlikely that a fair amount of local population extinction would be expected.

Several questions still exist that can only be answered with more study. First, the number of breeding adults present in these ponds could be determined directly by a mark-recapture study, or at least an estimate of the number of breeding females could be obtained by counting the number of egg masses present in a given pond. This was not possible in this study, as the eggs had already hatched by the time that the breeding ponds were identified and sampled. If the number of breeding adults were low then this would strengthen the case for drift playing a powerful role in shaping the genetic variation in this system.

To further investigate the observed heterozygote deficiency at four of the examined loci more microsatellite loci could be developed for this species. More loci could reveal whether the pattern of heterozygote deficiency continued to be present at new loci or if the high percentage of current loci with heterozygote deficiency is due to the small number of loci examined. Also sampling over several years could determine whether the low observed heterozygosity persists or is transient in nature.

Similarly, sampling over several years could also show whether the high Fst and Rst values observed at several of the pairs of breeding ponds separated by short distances are transient in nature or whether they persist over time. If these values tended to

diminish over time this would add credence to the idea that these are transient founder effects and will be attenuated over time due to occasional gene flow.

Finally, the pattern of isolation by distance that was strongly suggested by the current data could be more closely examined by adding more sampling sites at intermediate and long-range interpond distances.

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