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Genetic diversity and genetic structuring at multiple spatial scales across the range of the Northern Leopard Frog, Rana pipiens

Ryan P. O'Donnell
Utah State University

GENETIC DIVERSITY AND GENETIC STRUCTURING AT MULTIPLE SPATIAL

SCALES ACROSS THE RANGE OF THE NORTHERN LEOPARD FROG,

RANA PIPIENS

by

Ryan P. O'Donnell

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of

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Approved:

_____	_____
Karen E. Mock	Karen H. Beard
Major Professor	Committee Member
_____	_____
Edmund D. Brodie, Jr.	S. K. Morgan Ernest
Committee Member	Committee Member
_____	_____
David N. Koons	Mark R. McLellan
Committee Member	Vice President for Research and
	Dean of the School of Graduate Studies

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ABSTRACT

Genetic Diversity and Genetic Structuring at Multiple Spatial Scales across the Range of

the Northern Leopard Frog, *Rana pipiens*

by

Ryan P. O'Donnell, Doctor of Philosophy

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Major Professor: Karen E. Mock
Department: Wildland Resources

Despite a thorough understanding of the proximate mechanisms that drive genetic

diversity, we are still very poor at predicting the genetic diversity of natural populations.

Understanding patterns of genetic diversity is important for many reasons, including

predicting species' adaptation to climate change and predicting the spread of invasive

species, but it is particularly important for species that are declining. This dissertation

attempts to explain patterns in genetic diversity at multiple spatial scales across the range

of the Northern Leopard Frog, *Rana pipiens*, which is declining across large portions of

its range.

Genetic diversity is often lower in edge populations than in central populations.

Genetic diversity may be reduced in edge populations *per se*, or populations that occur at

the edge of the species' range may have low diversity because they have recently

expanded into new habitat and thus show signs of founder effects. In Chapter 2, we

tested several alternative hypotheses to explain genetic diversity across the species'

range, and to explain why some edge populations may not show reduced genetic diversity. We found that genetic diversity was reduced in edge populations relative to central populations, but was not reduced in populations in previously glaciated areas relative to previously unglaciated areas; therefore position at range edge had a stronger effect in reducing diversity than recent colonization of new habitat. We found that genetic diversity declined linearly towards the range edge in one of two transects from range center to range edge. We concluded that genetic diversity in this species is generally reduced by position at the range edge, but that this effect may differ among edges.

In Chapter 3, we tested the hypothesis that eastern and western populations were genetically distinct. We found two distinct clades that introgress in some markers but are distinct and defined by narrow boundaries in the eastern Great Lakes region in others. We concluded that genetic diversity in the Mississippi River region was elevated by the introgression of descendants from two Pleistocene refugia.

In Chapter 4, we analyzed genetic diversity within populations throughout Arizona to assess potential source populations for reintroductions. We also analyzed mitochondrial DNA to determine whether any populations contained genetic material not native to the region. Populations in one area had high genetic diversity and high gene flow among populations, but also contained evidence of introduction of eastern frogs. We conclude that supplementing genetic diversity in other populations with translocations from this area is not recommended.

(133 pages)

PUBLIC ABSTRACT

Genetic Diversity and Genetic Structuring at Multiple Spatial Scales across the Range of

the Northern Leopard Frog, *Rana pipiens*

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Genetic diversity is the raw material for evolution: evolution cannot happen

without genetic diversity, and the ability of a population to respond to a changing

environment depends directly on how diverse its genes are. Understanding the

distribution of genetic diversity is important for many reasons, including predicting

whether species will be able to adapt to climate change and predicting the spread of

invasive species. Information about the distribution of genetic diversity across the range

of the Northern Leopard Frog, a declining species, will not only help us to ensure that the

species can continue to evolve in response to changing environmental conditions, but it

will also help us gain a better understanding of what factors drive genetic diversity in

populations of other species. In Chapter 2, we found that genetic diversity was reduced

in edge populations relative to central populations, but was not reduced in populations in

previously glaciated areas; therefore position at range edge had a stronger effect in

reducing diversity than recent colonization of new habitat. In Chapter 3, we found two

distinct lineages within the species that mix in the eastern Great Lakes region, elevating

genetic diversity in that area. In Chapter 4, we found that populations in the Stoneman

Lake area of Arizona had high genetic diversity, but also contained evidence of

introduction of eastern frogs, and we concluded that moving frogs from the Stoneman

Lake area to restore diversity in other Arizona populations is not recommended.

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CHAPTER 1

INTRODUCTION

Populations vary across species' ranges in many ways. Populations at the edge of

the range may encounter competitors, parasites, or predators not encountered in the

central part of the range, may be under greater physiological stress due to climatic

conditions at the limit of the species' tolerance, and may be smaller and more isolated

from one another (reviewed in Holt and Keitt 2000; Gaston 2003). When these

differences result in reduced gene flow, increased genetic drift, or stronger selection in

edge populations, as they often do, these effects may act to reduce genetic diversity in

peripheral populations and to increase their divergence from one another (Gaston 2003).

The prevailing paradigm is that genetic diversity is lower at species' range edges,

and higher at range centers (Gaston 2003; Eckert et al. 2008), predicted primarily from

the metapopulation dynamics which often affect populations at range edges (Holt and

Keitt 2000). As populations become smaller, more isolated, and more ephemeral towards

the edge of a species' range, genetic diversity is expected to be reduced due to genetic

drift. However, many studies have found that this is not always the case. A 2003 review

found that 39% of 36 studies did not find reduced diversity in edge populations (Gaston

2003). A later review came to a nearly identical conclusion, finding that 36% of 134

studies did not demonstrate reduced diversity in edge populations (Eckert et al. 2008).

The difference in the number of studies included in these two reviews separated by only

five years gives an accurate impression of the rate at which this body of literature is

growing. However, we still lack the ability to predict when genetic diversity will decline,

or not decline, towards the edge of a species' range. Despite a broad range of studies

describing the pattern of genetic diversity across species' ranges, there has been little

effort to test alternative explanations for the patterns observed (Eckert et al. 2008).

Understanding the patterns of genetic diversity across species' ranges is important

for many reasons. For example, as our planet warms, species' ranges are expected to

shift up in elevation and towards the poles (e.g. Davis and Shaw 2001). Knowing the

patterns of genetic diversity across a range can help us to predict the ability of these

species to adapt or shift their ranges in response to climate change (Pujol and Pannell

2008; Pearson et al. 2009). Second, modern epidemiology is often concerned with the

spread of pathogen genotypes through a host population. Knowing how genetic diversity

varies across the ranges of hosts and pathogens is important for planning responses to

emerging diseases and for predicting their spread and evolution (e.g. Levis et al. 1998;

Biek et al. 2003). Third, one of the greatest threats to biodiversity is the spread of

invasive species (Wilcove et al. 1998). Similarly to the case of spreading diseases,

understanding patterns of genetic diversity at invasion fronts will likewise enhance our

ability to anticipate and manage the spread of invasive species (Prentis et al. 2008; Kolbe

et al. 2010; Lawson Handley et al. 2011).

Understanding the factors driving genetic diversity across the range is important

for many species, including the Northern Leopard Frog, *Rana pipiens*. This species is

found across much of North America, ranging from the Atlantic coast west to eastern

Washington State and northeastern California, and from Arizona and New Mexico in the

south, north to the Northwest Territories of Canada (Rorabaugh 2005). It can be

abundant where it is found, contributing significantly to the vertebrate biomass of some

ecosystems and linking aquatic and terrestrial habitats (Merrell 1968; Harding 1997). It

is also an economically and culturally important species, used as fishing bait, kept as

pets, used for dissections in education, and used for medical research (Gibbs et al. 1971).

Perhaps in part because of the wide distribution and sometimes high local

abundance of the Northern Leopard Frog, when population declines and range

contractions were first noted in the species in the 1970s, they were unexpected (Gibbs et

al. 1971). Over the subsequent decades, population losses continued to be documented

across the species' range. Local extinctions were noted, for example, in the mountains of

Colorado in the 1970s and early 1980s (Corn and Fogleman 1984), in several areas of

Wyoming in the late 1970s and early 1980s (Baxter and Stone 1985), and in Arizona in

the mid-1980s (Clarkson and Rorabaugh 1989). Losses of populations continue to be

documented across the species' range (e.g. Werner 2003; Germaine and Hays 2009;

Desroches et al. 2010).

Declines were noted not only in the number and distribution of populations, but

also in abundance. For example, in the central part of the species' range in Wisconsin,

declines in abundance were first noted in the 1970s (Hine et al. 1981) and have continued

to at least the late 1990s (Mossman et al. 1998), and in Indiana a 45-year study

documented significant reductions in numbers (Minton 1998). In Iowa, it was estimated

that total population numbers of Northern Leopard Frogs had declined by two to three

orders of magnitude since the turn of the 20th century (Lannoo et al. 1994).

In response to population declines throughout the Northern Leopard Frog's range,

but especially in the west, many agencies have begun to plan for and implement

management actions intended to restore the species to previously occupied parts of its

range. For example, Alberta has an active relocation program and is attempting to restore the species in areas where it was once found (Wilson et al. 2008). Likewise, the Arizona Game and Fish Department has begun conducting some translocations and is planning more (S. MacVean, Arizona Game and Fish Department, pers. comm.). These and other management actions will directly benefit from information about the distribution of genetic diversity within this species and from information about the relatedness of populations to one another.

The applications of the findings presented in this dissertation are not limited to this species alone. Many different kinds of management actions are motivated by the goal of maximizing the likelihood of population persistence (Brook et al. 2000; Morris et al. 2002; Reed et al. 2003), and population persistence depends on having adequate genetic diversity to allow adaptation in response to changing conditions and to avoid the negative effects of inbreeding depression. Unfortunately, directly measuring genetic diversity is expensive, and our predictions of population genetic diversity are imperfect. Although it is clear that population genetic diversity is a function of the processes of mutation, selection, migration, and drift, integrating these processes into the prediction of current genetic diversity in any natural population remains quite difficult. The general theme of this dissertation is to develop a greater understanding of the processes that drive genetic diversity in natural populations, with the dual goals of improving our ability to predict genetic diversity across species' ranges generally and providing more insight into the phylogenetic history and genetic diversity of the Northern Leopard Frog specifically.

My dissertation will attempt to explain some of the patterns in genetic diversity across the range of the Northern Leopard Frog. I will approach this broad issue in the

following three chapters. In Chapter 2, I will test competing explanations for reduced genetic diversity at range edges. Genetic diversity can be reduced in edge populations by position on the edge *per se*, or by founder effects in recent colonization of newly available habitat, but these two confounding explanations are rarely addressed. I will study genetic diversity across the range of the Northern Leopard Frog first to test whether genetic diversity is predicted by position at range center or edge, and second to test whether diversity is predicted by the history of the population (position above or below the glacial maximum in the last ice age). Most previous studies of genetic diversity used categorical approaches similar to what I use in this first part, but because of this, we have little understanding of how diversity changes between the center and edges of ranges. Accordingly, I will examine the pattern of genetic diversity continuously from range center to edge in two transects. The change in genetic diversity along these two transects will help indicate whether reductions in genetic diversity are predominately influenced by decreasing connectivity of populations (creating a constant decrease in genetic diversity), or by positive feedback loops, which are expected to result in a dramatic loss of diversity at a threshold of connectivity as reduced diversity reduces fitness, which reduces population size, which results in further losses of diversity (Gilpin and Soulé 1986).

In Chapter 3, I will assess the range-wide mitochondrial and nuclear phylogeography of the Northern Leopard Frog. Previous work has shown deep phylogenetic divergence between eastern and western Northern Leopard Frogs on the basis of mitochondrial DNA (Hoffman and Blouin 2004a), but mitochondrial DNA is only a very small part of the genome, and it is inherited differently from the rest of the genome, having an effective population size that is four times smaller than that of rest of

the genome and being inherited through females only. I will analyze four nuclear sequences and seven nuclear microsatellites to test whether the differences between eastern and western frogs that were identified by mitochondrial sequencing are also evident in nuclear DNA.

In Chapter 4, I will examine a specific case study of an edge population with unexpectedly high genetic diversity. Northern Leopard Frogs in the Stoneman Lake area of Arizona were shown in previous work to have relatively high genetic diversity (Kimberling et al. 1996), and they were considered as a potential source of frogs for translocations elsewhere in northern Arizona, where the species has dramatically declined in recent decades (Clarkson and Rorabaugh 1989). Hoffman and Blouin (2004a) found one individual in their sample of ten from that population that had an eastern mitochondrial haplotype, far from the nearest eastern populations. They speculated that they might have happened upon an eastern frog that was a recently released pet or laboratory animal. I will investigate this population, in the context of others in the area, to determine whether the high diversity there may be explained by the successful establishment of eastern frogs in an area where western frogs are native.

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CHAPTER 2

WHEN AND HOW DOES GENETIC DIVERSITY DECLINE TOWARDS A RANGE

EDGE? TESTING ALTERNATIVE HYPOTHESES OF THE PROCESSES DRIVING

GENETIC DIVERSITY IN POPULATIONS[2]

Abstract

An understanding of the factors driving genetic diversity in populations is
increasingly important in many areas of biology, including epidemiology, biological
consequences of climate change, and the spread of invasive species. However, despite
hundreds of papers studying genetic diversity across species' ranges, we lack a cohesive
view of the factors driving that diversity. Few papers have empirically addressed
alternative explanations for genetic diversity, and even fewer have incorporated
information on the history of populations or include assessments of diversity
continuously, rather than categorically. Genetic diversity can be reduced in edge
populations by position on the edge *per se* (i.e. by factors inherent to range edges), or by
founder effects in recent colonization of newly available habitat, but these two
confounding explanations are rarely addressed. We studied genetic diversity across the
range of the Northern Leopard Frog, first to test whether genetic diversity is predicted by
position at range center or edge, and second to test whether diversity is predicted by the
history of the population (position above or below the glacial maximum in the last ice
age). A second shortcoming of many studies available on the distribution across species'
ranges is that they use only a categorical approach, as we did in the first part, and thus

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[2] This chapter is co-authored by Ryan P. O'Donnell and Karen E. Mock.

little is known about how diversity changes from range center to edge. Accordingly, we examined the pattern of genetic diversity continuously from range center to edge in two transects. In the first part, we found that genetic diversity was reduced in edge populations relative to central populations, but was not reduced in populations in glaciated areas versus unglaciated ones, providing evidence that position at range edge has a stronger effect in reducing diversity than recent colonization in this species. In the second part, we found that genetic diversity declined linearly towards the range edge in one transect, but did not decline in the other. These transects differed in the nature of the range edges they included: a transect with increasingly isolated populations showed a decline in diversity, and a transect through continuous habitat did not. Taken together, these results provide evidence that genetic diversity in this species is reduced by position at the range edge, but that these effects may not be evident in all edges.

Introduction

An often cited "rule" of population genetics is that genetic diversity tends to be higher in the interior of species' ranges and lower at the periphery (e.g. Hutchison 2003; Gapare and Aitken 2005; Arnaud-Haond et al. 2006; Lind et al. 2007). This may be directly related to the abundant center distribution (reviewed in Sagarin and Gaines 2002): if populations are smaller at the periphery, they may be more susceptible to loss of alleles through genetic drift (Vucetich and Waite 2003) and to stochastic extinction and recolonization (Caughley 1994), reducing genetic diversity through repeated founder effects (Holt and Keitt 2000). Edge populations may further have less gene flow to counteract the stochastic loss of alleles because they can only receive immigrants from

one direction (García-Ramos and Kirkpatrick 1997; Rowe and Beebee 2001).

Populations at the edge of the range may also lose diversity through selection if they

encounter unique selective pressures, such as competitors, parasites, or predators not

present in the central part of the range, or climatic conditions at the limit of the species'

tolerance (Gorodkov 1986; Holt and Keitt 2000; Gaston 2003). All of these varied

processes may combine to reduce genetic diversity symmetrically within all peripheral

populations around the edge of a species' range (Fig. 2-1A).

However, an additional explanation for reduced diversity at range edges may be at

work, which would not usually affect all range edges equally (Fig. 2-1B). If ranges shift

in space, populations in the expanding part of the range may harbor reduced genetic

diversity due to founder effects, a special case of reduced population size (e.g. Green et

al. 1996; Austin et al. 2004a). Founder effects could reduce genetic diversity in

peripheral populations as well as populations that are currently geographically central, if

both were part of a recent range expansion. If founder effects are more prevalent than

processes affecting peripheries *per se*, it may explain why more than one third of range

peripheries do not have reduced genetic diversity relative to central parts of the range

(Eckert et al. 2008) if those peripheries were not part of a recent range expansion.

Most of the studies that have attempted to investigate causes of reduced diversity

at the periphery of species' ranges have been concerned with distinguishing between

anthropogenic and prehistoric reductions of diversity (e.g. Matocq and Villablanca 2001;

Hoffman and Blouin 2004b). When reduced diversity in peripheral populations is found

to be a result of prehistoric processes, either through comparison with reference

populations or with museum specimens, there is often little further investigation into the

causes of reduced genetic diversity at the periphery. In two exceptions, genetic

diversity was found to follow a gradient declining from a presumed glacial refugium to

the most recently established populations, supporting the idea that range expansion, not

periphery *per se*, was responsible for reducing genetic diversity in peripheral populations

(Garner et al. 2004; Gapare and Aitken 2005). In two other cases, a similar pattern of

reduced diversity in edge populations was explained by reduced population sizes in the

marginal habitats inhabited by peripheral populations (Johansson et al. 2006; Lind et al.

2007). More studies are needed that test alternative hypotheses explaining diversity in

edge populations (Gaston 2003).

A further limit on many previous studies of the factors driving population genetic

diversity is that most studies use only a categorical approach. Over two thirds of studies

in a recent review have considered only discrete categories of population types, "edge"

vs. "center" (Eckert et al. 2008). This approach reveals little about the manner in which

diversity declines towards a range edge. The gradual breakup of populations towards the

range edge as depicted, for example, by Gorodkov (1986) suggests that a decline in

diversity should occur gradually. However, reduced genetic diversity can be part of a

series of positive feedback loops which may result in an abrupt decline in diversity at a

threshold of isolation among subpopulations (Gilpin and Soulé 1986). For example, if

populations are smaller and more isolated at the periphery, they may be more susceptible

to genetic drift (Vucetich and Waite 2003) and to stochastic extinction and recolonization

(Caughley 1994), reducing genetic diversity through repeated founder effects. Reduced

genetic diversity may in turn make a population more susceptible to extinction. This

positive feedback between extinction rate of isolated populations and reduced genetic

diversity is one way in which genetic diversity could decline abruptly at a threshold of population isolation. However, more studies using a continuous sampling strategy (rather than categorical) are needed to test whether genetic diversity declines linearly or with a threshold towards range peripheries.

An additional pattern in genetic diversity from range edge to range center has been described, where diversity peaks at an intermediate optimum level of isolation. This pattern was found in one of very few other studies of genetic diversity along a transect (Kark et al. 2008). Kark et al. speculated that the intermediate maximum in diversity they found might result from a balance between bottlenecks that reduce genetic diversity in small edge populations, and large effective population sizes in the range center that make the establishment of new, rare alleles unlikely. This hypothesis of an intermediate optimum "goldilocks zone," where populations are small and isolated enough to be likely to retain new mutations, but also connected enough to gain diversity through gene flow, likewise requires additional testing.

We investigated patterns of genetic diversity around the range of the Northern Leopard Frog, *Rana pipiens*, to test two hypotheses for predicting levels of genetic diversity across species' ranges. First, we tested the hypothesis that historic shifts in range have affected the current distribution of genetic diversity across the range of the species (Fig. 2-1B), against the alternative hypothesis that position at the edge of the range affects genetic diversity directly, without consideration of shifts in the species range through time (Fig. 2-1A). Second we tested the hypothesis that genetic diversity declines linearly towards range edges, against the alternatives that it declines abruptly at a threshold or that it has an intermediate optimum. We tested our first hypothesis by

sampling populations from around the range of the species, selecting populations that were at range edge or range center, and that were covered by ice at the most recent glacial maximum or colonized since that glacial maximum. We tested our second hypothesis by sampling population genetic diversity across two transects in the range of the Northern Leopard Frog, to test whether genetic diversity declines linearly, as predicted by the gradually increasing isolation between populations, or in a threshold, as predicted by the positive feedback mechanisms of Gilpin and Soulé (1986), or an intermediate optimum (Kark et al. 2008).

The Northern Leopard Frog is particularly well suited for a study of the factors affecting population genetic diversity. Its range covers much of North America (Rorabaugh 2005), so differences in diversity between interior and peripheral populations are expected to be more dramatic than for a species with a small range, where the entire range may have low levels of diversity. Second, a range-wide molecular phylogeny based on mitochondrial (Hoffman and Blouin 2004a) and nuclear sequences (Chapter 3) is already available for this species, allowing confirmation of the history of populations around the range from two marker types with different modes of inheritance. Understanding the causes of reduced genetic diversity in peripheral populations requires knowledge of the history of those populations, but such knowledge has almost never been incorporated in studies of genetic diversity across species' ranges (Eckert et al. 2008). Third, about half of the range of this species was covered by the last glacial maximum, providing a convenient distribution of population histories for testing the effects of range expansion on genetic diversity. Finally, populations of this species are increasingly

isolated towards the range edge (Kimberling et al. 1996; Rorabaugh 2005), so they fit

the predictions for a case where diversity might decline towards the range edge.

Materials and methods

We collected genetic tissue from Northern Leopard Frogs around their range in

the summers of 2008-2010, sampling across the range in both glaciated and unglaciated

populations and in edge and central populations (Fig. 2-2), plus two transects from range

center to edge (Fig. 2-3). In addition, we acquired previously collected samples from

additional populations, the oldest collections being from 2004. Populations used for the

two transects stretched from the edge of the species' range in Arizona to the center in

Montana, and from Montana to another edge in Nebraska (Fig. 2-3). In total, we

analyzed genetic data from 989 frogs from 42 populations (mean 23.5 frogs per

population, range 14-34). In the categorical analysis, we analyzed 567 samples from 24

populations; in the transects, we analyzed 586 samples from 25 populations. Samples

from 164 individuals and seven populations were used in both analyses.

Tissue samples were collected by clipping the tip of the third toe on the right hind

foot directly into a microvial containing 95% ethanol. When possible, we collected tissue

samples from freshly road-killed specimens. To minimize the risk of infection, we

applied an antibiotic/anesthetic ointment to the cut toes of live sampled frogs before

releasing them at their point of capture (Green 2001). Surgical instruments were

sterilized between each frog to eliminate the risk of sample contamination and to reduce

the risk of spreading diseases among frogs. Samples were frozen and stored at -80°C

until DNA extraction. DNA was extracted from toe tips using a standard chloroform

extraction (Müllenbach et al. 1989). The purified DNA was resuspended in a Tris-
EDTA buffer (0.1M Tris, 0.1M EDTA, pH 9.0) and stored at -80° C until use.

We amplified seven microsatellite loci: Rpi100, Rpi101, Rpi102, Rpi104, Rpi107,
and Rpi108 (Hoffman et al. 2003); and RP193 (Hoffman and Blouin 2004b) in all
samples. Each 25 mL polymerase chain reaction (PCR) contained 0.4 µmol/L of each
primer, 150 µmol/L dNTPs, 1× standard PCR buffer (including 1.5 mmol/L $MgCl_2$), 2.5
units of Taq polymerase, and ~50 ng of genomic DNA. PCR conditions were 2 minutes
of initial denaturation at 95° C, followed by 30 cycles of the following steps: 95° C for 30
seconds, annealing temperature for 30 seconds, and 72° C for 1 minute; followed by a 10
minute final extension at 72° C. Locus-specific annealing temperatures were as follows:
Rpi100: 52° C, Rpi101: 62° C, Rpi102: 50° C, Rpi103: 55°C, Rpi104: 56° C, Rpi107:
52° C, Rpi108: 52° C, and RP193: 56° C. The PCR product was visualized on a 0.7%
agarose gel to check for product quantity and size. PCR products were then analyzed on
an ABI 3100 or 3730 sequencer.

Microsatellite data was used to calculate two estimates of neutral genetic
diversity. Allelic richness was calculated as the number of alleles present in each
population per locus, rarified to adjust all sample sizes to the smallest population in our
sample (14) using HP-RARE (Kalinowski 2005). Gene diversity (mean expected
heterozygosity) was calculated for each locus, using FSTAT (Goudet 1995), then
averaged across loci.

To test whether glacial history or position on the range affected genetic diversity,
gene diversity and allelic richness were used as response variables in separate t-tests.
Because we had a priori expectations for the direction of the difference, with lower

measures of diversity expected in glaciated populations and edge populations, we report one-tailed p-values. To help visualize genetic diversity across the range, we also produced a map of interpolated genetic diversity for each measure, gene diversity and allelic richness, using inverse distance weighting in ArcGIS 10.0.

For the transect analysis, allelic richness and gene diversity were plotted as a function of geographic distance from the nearest range edge. We used program SegReg (Oosterbaan 1994) to test whether diversity changed linearly or in a threshold fashion from range center to edge; we also tested the fit of a quadratic regression. We predicted that if genetic diversity does not decline towards the range edge, then a regression of diversity on distance to range edge would have a slope that was not significantly different from zero. If genetic diversity declined linearly towards the range edge, then a regression of diversity on distance to range edge would have a significant linear relationship. If genetic diversity declined in a threshold function towards the range edge, then a regression of diversity on distance to range edge would have a relationship that was described significantly better by a two different linear regressions with zero slope than a linear regression. If genetic diversity showed an intermediate optimum, a quadratic regression would fit the data better than any linear or threshold model. Significance for improvement of fit between models was tested with Akaike's Information Criterion (AIC).

Results

Categorical analysis

Both gene diversity and allelic richness tended to be lower in edge populations

than in center populations, although the difference was marginally nonsignificant (gene

diversity: P = 0.07; allelic richness: P = 0.06; Fig. 2-4). There was no significant

difference in gene diversity or allelic richness between populations in glaciated versus

unglaciated regions (gene diversity: P = 0.15; allelic richness, P = 0.36; Fig. 2-4). A map

of interpolated genetic diversity across the range showed lower diversity in both

measures in the west, with a peak in genetic diversity in the center of the eastern portion

of the range (Fig. 2-5).

Transect 1

Both measures of genetic diversity were lower in edge populations than in center

populations for the first transect. Gene diversity and allelic richness were each best

explained by a single linear regression with no breakpoint (Fig. 2-6). The distance of a

population from the range edge was a good predictor of its gene diversity ($R^2 = 0.82$, P <

0.001) and its allelic richness ($R^2 = 0.86$, P < 0.001). Adding a breakpoint or a quadratic

term did not improve the fit of the model.

Transect 2

Neither measure of genetic diversity was lower in edge populations than in center

populations for the second transect. Gene diversity and allelic richness were each best

explained by a single linear regression with no slope (Fig 2-7). The distance of a

population from the range edge did not significantly correlate with its gene diversity (P =

0.22) or its allelic richness (P = 0.44). For gene diversity, adding a breakpoint

increased the fit of the model but not significantly so ($F_{1,7}$ = 2.15, P = 0.19), and a

quadratic term did not improve the fit. For allelic richness, neither a breakpoint nor a

quadratic term increased the fit of the model.

Discussion

There are many alternative explanations for the factors that drive genetic

diversity, and these explanations are not necessarily mutually exclusive. In this paper, we

used groups of populations to test two alternative explanations for driving genetic

diversity. We then tested for thresholds or intermediate optima in genetic diversity along

two transects from range center to range edge.

Genetic diversity was reduced more by a population's position at the edge of the

range than it was by post-Pleistocene founder effects. Using an alpha of 0.05, all of our

tests are nonsignificant, but both measures of genetic diversity were lower in edge

populations. Contrary to our predictions, both measures of genetic diversity were

actually higher in populations that were covered by the last glacial maximum, although

these differences did not approach statistical significance. Visual inspection of the maps

of interpolated genetic diversity reveals that the higher diversity in glaciated areas results

from high diversity generally in the eastern part of the range, which is primarily north of

the glacial maximum. From these results, we conclude that there is weak evidence that

position at the range edge *per se* reduces genetic diversity in this species, and no evidence

that post-Pleistocene founder effects persist in affecting modern genetic diversity in this

species.

Our tests for thresholds or intermediate optima in genetic diversity along two transects from range center to range edge gave results that differed between the two transects. In the first transect, we found that genetic diversity declined linearly towards the range edge. The results from this transect do not support a stepwise decline in diversity predicted from positive feeback loops reducing diversity, nor an intermediate optimum in diversity as described by Kark et al. (2008).

In the second transect, diversity did not change significantly towards the range edge. This lack of a significant change in genetic diversity could be due to at least two different mechanisms. First, this second transect was shorter than the first, and may have simply failed to show a pattern in genetic diversity because sampling was not across a long enough transect for changes in diversity to be evident. Second, and probably more importantly, the nature of the edges of the range differ between these two transects. The first transect ends at the edge of a plateau, where populations are sparse and isolated (Chapter 4), and where suitable habitat largely ends. In contrast, the end of the second transect is formed by parapatry with a congeneric species, *Rana blairi*. Habitat suitable for leopard frogs is continuous throughout and beyond our transect, and is not increasingly fragmented as for the first transect.

The differences between these two transects further emphasize the importance of historical and biological context in predicting the genetic diversity of edge populations. For example, Howes and Lougheed (2008) found that genetic diversity in a North American lizard was best predicted by local climate, but with distance from range edge and an interaction between history and edge also contributing significantly to their model. This interaction, they speculated, was the result of edge populations in the recently

colonized part of the range not yet reaching drift-gene flow equilibrium. Thus position in the range interacted with population history to affect current levels of genetic diversity.

Understanding the patterns of genetic diversity at range edges has many important implications. For example, as our planet warms, species' ranges are expected to shift up in elevation and towards the poles (e.g. Davis and Shaw 2001). Knowing the distribution of genetic diversity at range edges can help us predict the ability of these species to adapt or shift their ranges in response to climate change, although more studies are needed to test whether geographic trends in neutral markers accurately predict congruent trends in phenotypic trait variation (Westphal et al. 2011). Second, modern approaches to epidemiology are often concerned with modeling the spread of pathogen genotypes through a host population (e.g. Levis et al. 1998; Biek et al. 2003). Knowing how genetic diversity is distributed at the edges of a pathogen's or host's range is important for modeling and predicting the spread of emergent diseases. Third, one of the greatest threats to biodiversity is the spread of invasive species (Wilcove et al. 1998). Similar to spreading diseases, understanding patterns of genetic diversity at invasion fronts will likewise enhance our ability to predict and manage the spread of invasive species (Sexton et al. 2002; Kolbe et al. 2010). Finally, being able to predict genetic diversity across species' ranges can help direct management and conservation efforts at populations with lower than expected diversity. For these and many other reasons, it is critical that more studies use continuous approaches and test alternative hypotheses to study the factors driving genetic diversity in populations.

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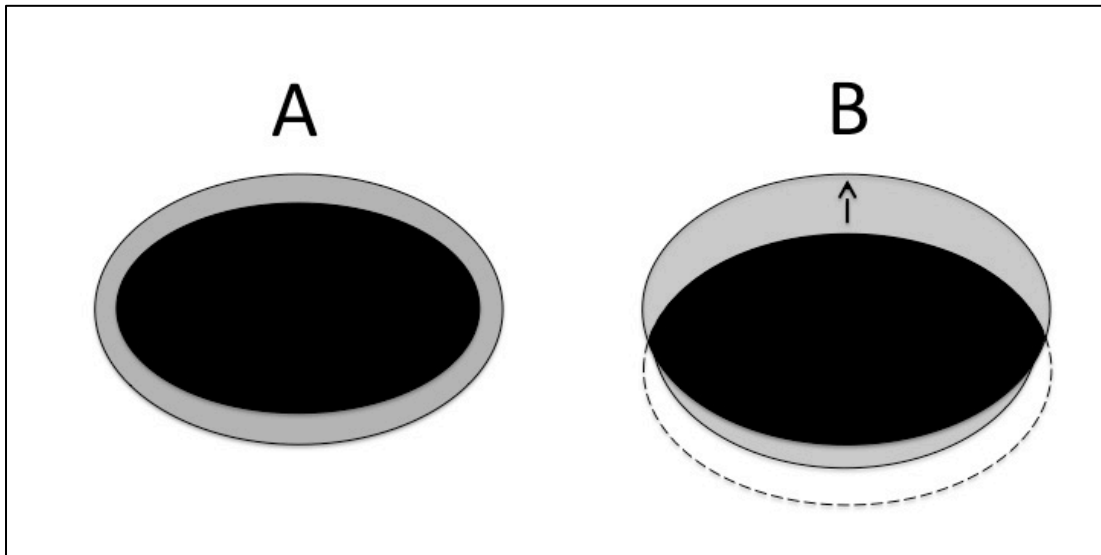
Fig. 2-1: Two simplified explanations for reduced genetic diversity at range edges. High

diversity areas of the range are shown in black, and low diversity in gray. A: Position at

the edge of the range *per se* reduces genetic diversity, usually through reduced

connectivity of populations and small population sizes in the periphery, resulting in

generally symmetric reductions in diversity around the range. B: Shifts in range cause

reduced diversity, usually through founder effects (a special case of small population

size) at the leading edge of the range expansion, leading in asymmetric reduction of

genetic diversity.
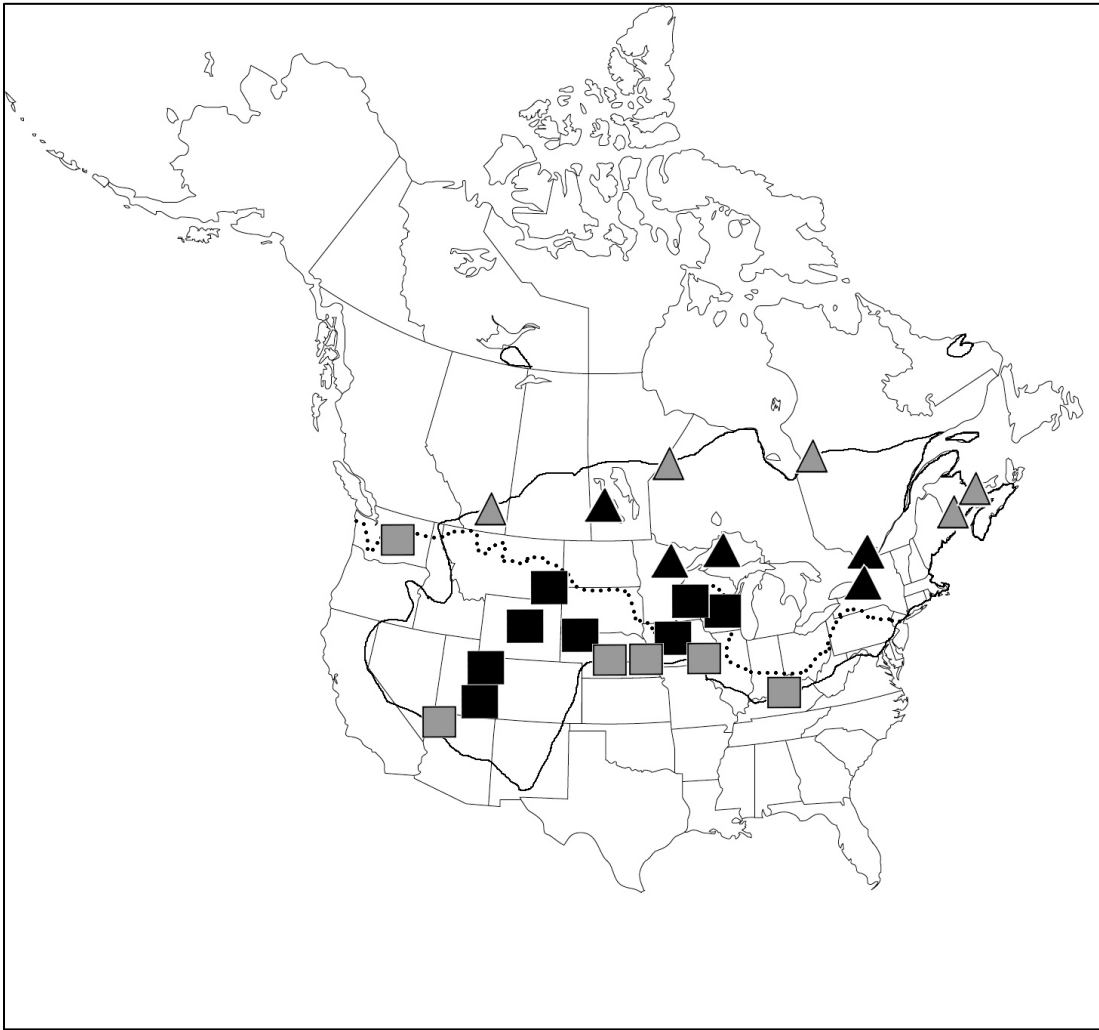
Fig. 2-2: Map of populations used in the categorical comparison of population types.

Squares represent unglaciated populations (areas not covered by the Wisconsonian

Glacial Maximum, n = 14); triangles represent glaciated populations (colonized since the

Wisconsonian Glacial Maximum, n = 10). Gray symbols indicate edge populations (n =

11); black symbols represent central populations (n = 13). The dotted line marks the

approximate extent of the Wisconsonian glacial maximum (Ehlers et al. 2011). The solid

line marks the current extent of the species' range.
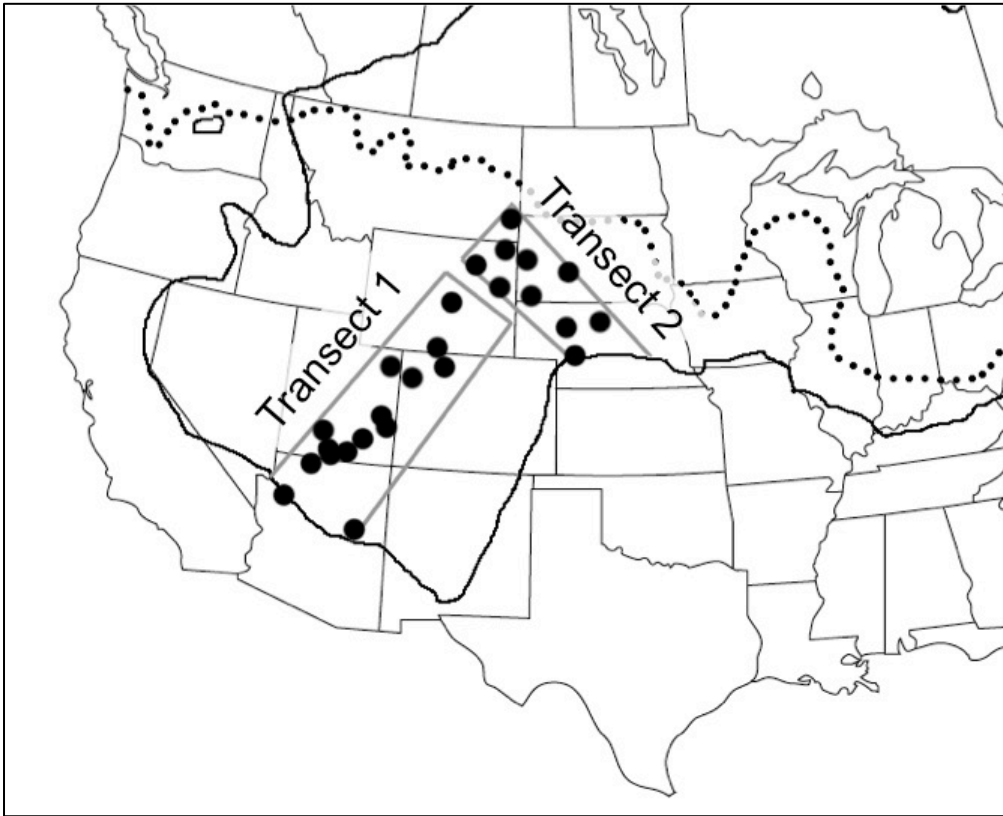
Fig. 2-3: Map of populations used in the transects in this study. Solid line represents the edge of the species' range. Dotted line indicates the extent of the Wisconsonian Glacial Maximum.
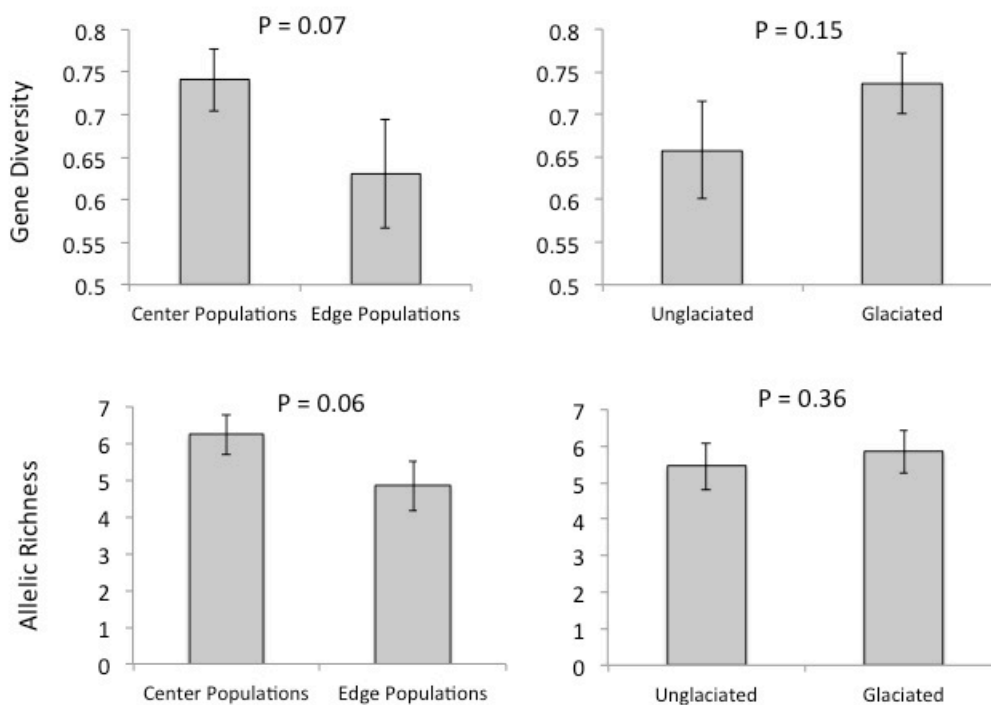
Fig. 2-4: Effects of two categories of position in the range on two measures of genetic diversity. Error bars indicate 95% confidence intervals of the mean (i.e., ± 2SE). See Fig. 2-2 for location of populations and sample sizes.
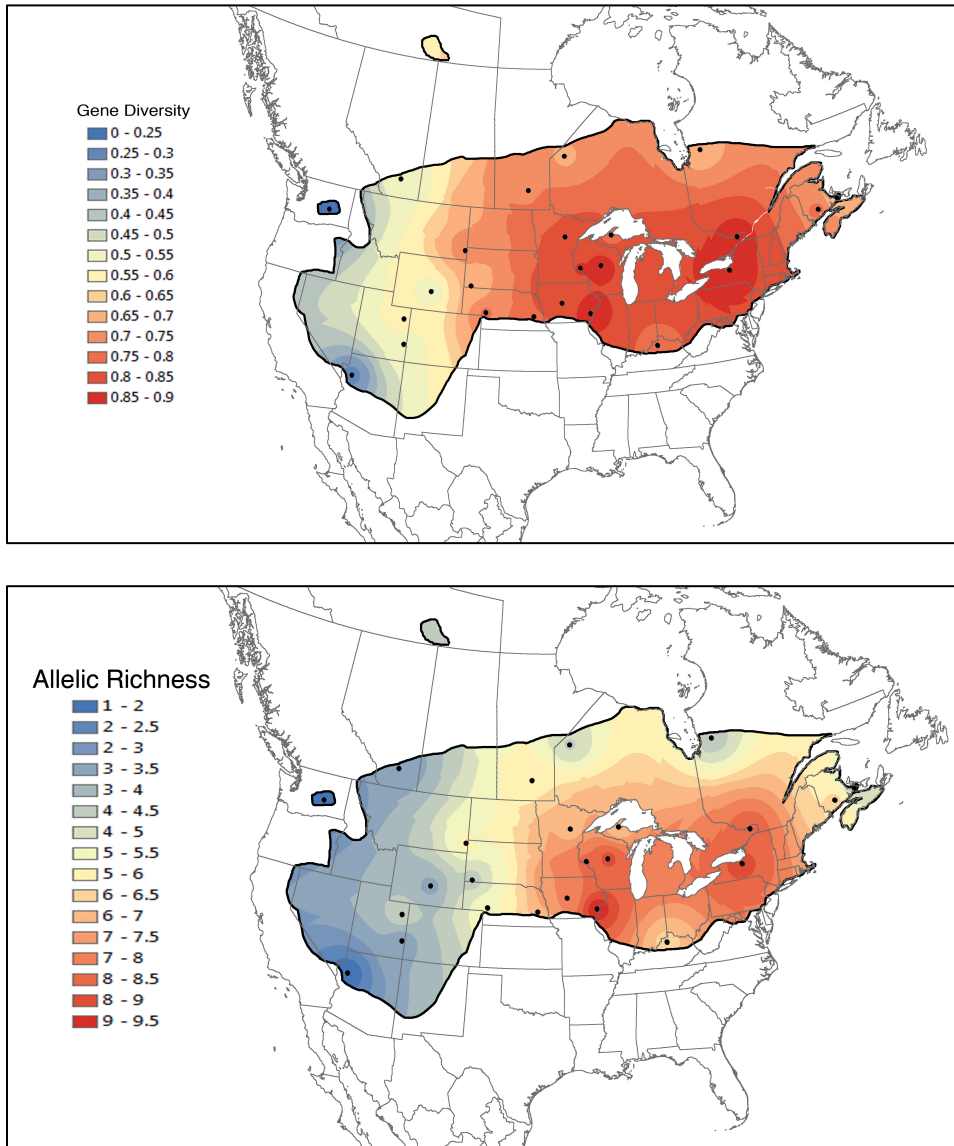
Fig. 2-5: Heat map of genetic diversity measures interpolated across the species' range, calculated using inverse distance weighting. Top panel shows gene diversity, bottom panel shows allelic richness.
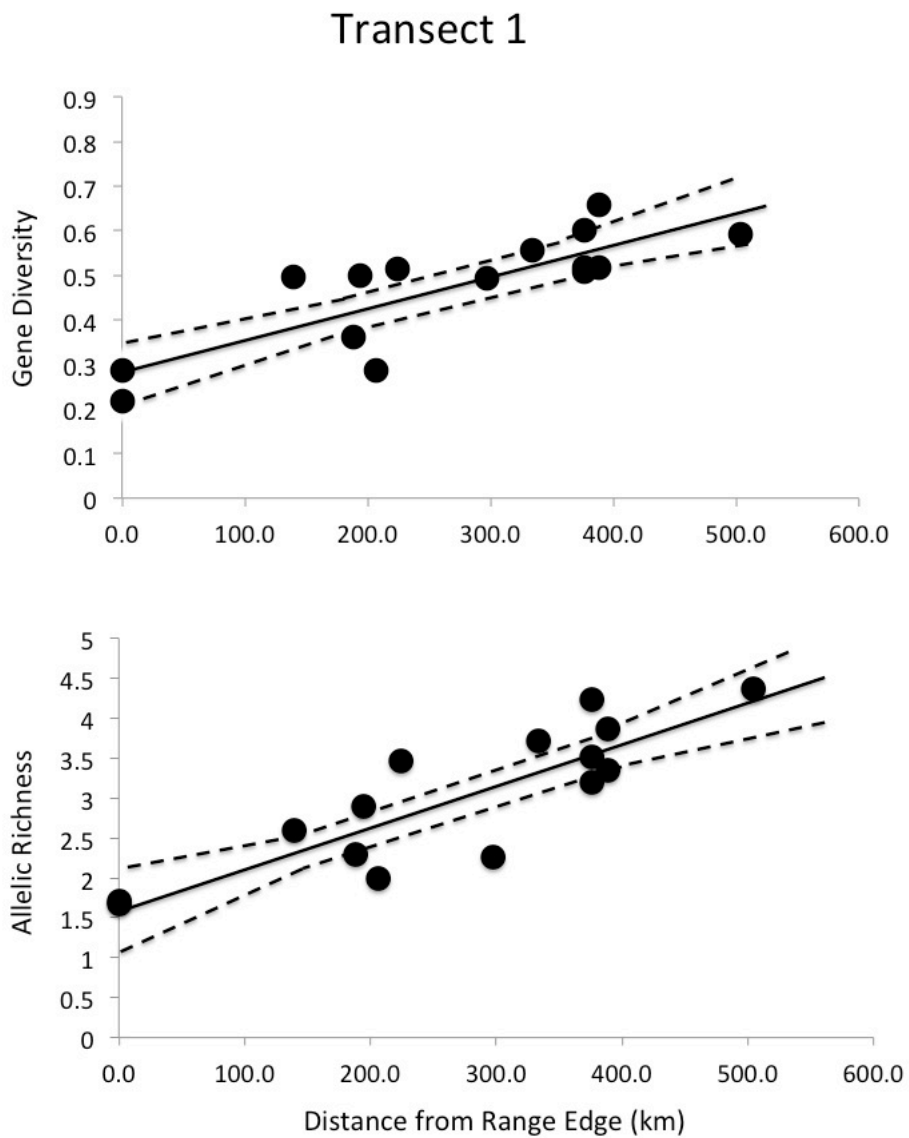
Transect 1

Fig. 2-6: Two measures of genetic diversity across the first transect from range edge (left) to center (right). Each point represents the genetic diversity of a population. Best fit lines (solid) and 95% confidence bands (dashed) were calculated in program SegReg.
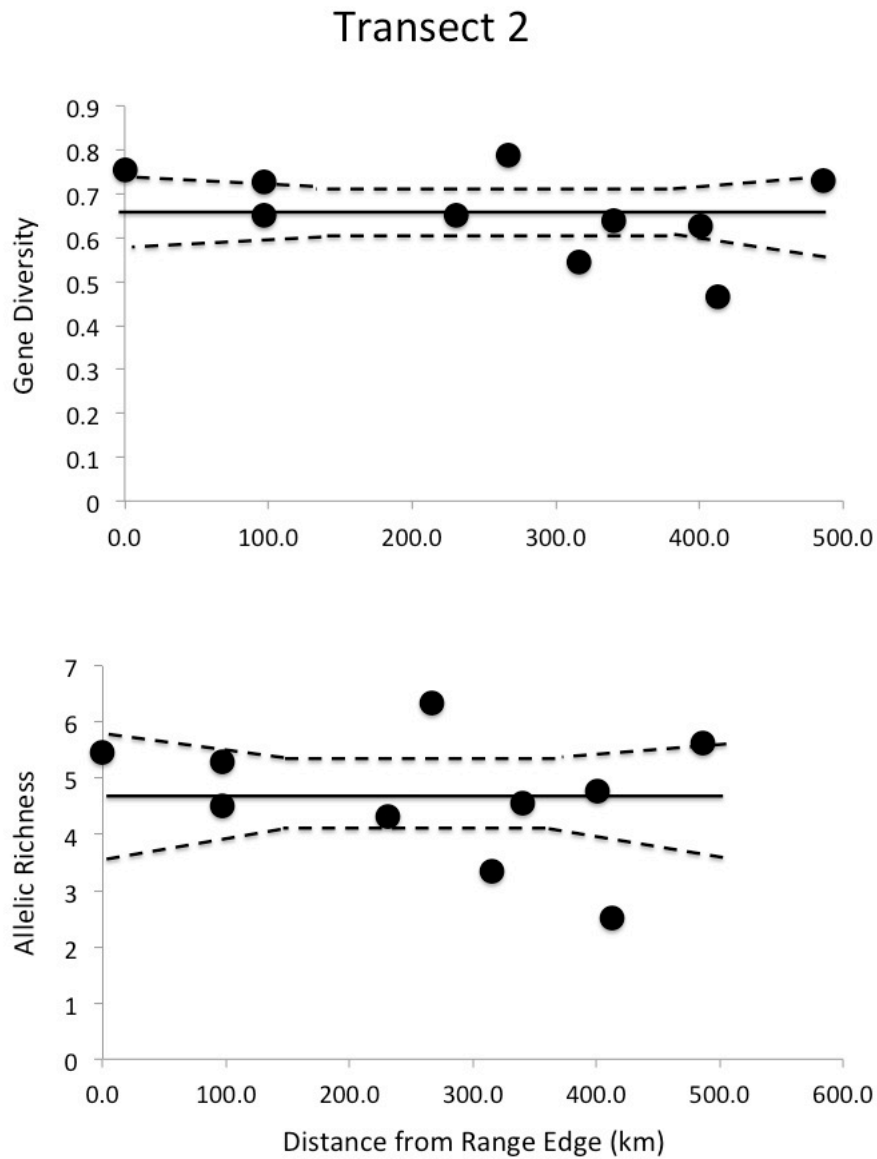
Fig. 2-7: Two measures of genetic diversity across the second transect from range edge (left) to center (right). Each point represents the genetic diversity of a population. Best fit lines (solid) and 95% confidence bands (dashed) were calculated in program SegReg.

CHAPTER 3

TWO FROG SPECIES OR ONE? A MULTI-MARKER APPROACH TO ASSESSING

THE DISTINCTIVENESS OF GENETIC LINEAGES IN THE NORTHERN

LEOPARD FROG, *RANA PIPIENS*[3,4]

Abstract

A genetic boundary at the Mississippi River, USA, has been suggested for the

Northern Leopard Frog, *Rana pipiens*, which was recently proposed for listing as

federally threatened in the western USA. This suggestion was made on the basis of

limited geographic sampling of a mitochondrial gene. However, mitochondrial DNA

represents a very small part of the genome and is not necessarily indicative of patterns in

nuclear DNA. We tested the hypothesis that eastern and western populations are

separated by a distinct genetic boundary by sequencing mitochondrial DNA more

extensively across the range, including focused sampling in the zone of hypothetical

introgression, and by analyzing four nuclear sequences and seven microsatellite loci. We

confirmed previous results that eastern and western populations have unique

mitochondrial sequences that are deeply divergent (3.8%) and which overlap only in a

narrow region around the Mississippi River. Nuclear sequences also show divergent

eastern and western lineages in some cases but with a broader zone of geographic

overlap. Microsatellite data correspond closely to mitochondrial data, differing between

east and west and changing abruptly near the Mississippi River. These data collectively

demonstrate that eastern and western clades of this species introgress considerably in

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[3] This chapter is co-authored by Ryan P. O'Donnell and Karen E. Mock.
[4] This chapter has been accepted for publication and is in press at Conservation Genetics.

some markers but are distinct and defined by clear and narrow boundaries in others.

We demonstrate that the Mississippi River forms an important, albeit somewhat

permeable, boundary between genetic lineages in this species. This genetic boundary

coincides with previously described discontinuities in morphological features.

Introduction

Despite the fact that leopard frogs, the "*Rana pipiens* group" (sensu Hillis and

Wilcox 2005), have been a model organism for studies of physiology and phylogenetics

for decades, species boundaries in this complex are still incompletely known. For

example, many species were only described in the 1970s and 1980s (e.g. Frost and

Bagnara 1976; Platz and Mecham 1979; Platz and Frost 1984), and species boundaries in

the Chiricahua Leopard Frog (*Rana chiricahuensis*) are still being resolved (e.g.

Goldberg et al. 2004; Hekkala et al. 2011). The most widespread member of the *Rana*

pipiens group is no exception: for over a century it has been debated whether the taxon

now known as the Northern Leopard Frog (*Rana pipiens*) is monomorphic or contains

two species with a border between them around the Mississippi River (e.g. Cope 1889;

Wright and Wright 1949; Dunlap and Platz 1981). Early descriptions of Northern

Leopard Frogs in the western part of the range described them as a separate subspecies

(*Rana pipiens brachycephala*) having a shorter head and no distinct barring on anterior

surface of the limbs, among other features (Cope 1889). Several authors were unable to

confirm Cope's morphological features (reviewed in Dunlap and Platz 1981), and

therefore Wright and Wright (1949) commented that most herpetologists of their time did

not consider Cope's *brachycephala* to be valid. However, Wright and Wright (1949)

considered the matter unsettled and summarized the work of others in showing the

range of *brachycephala* to include all of the western part of the range of *pipiens* to the

middle of Minnesota and Iowa. No further attempts were made to assess the validity of *R.*

p. brachycephala until Dunlap and Platz (1981) analyzed allozymes and calls and also

found no consistent difference between eastern and western Northern Leopard Frogs. By

this time, *R. p. brachycephala* was widely regarded as invalid.

The issue of whether eastern and western Northern Leopard Frogs were distinct

was revived by Hoffman and Blouin in 2004. In a range-wide phylogeny of the Northern

Leopard Frog, they detected two mitochondrial lineages that closely supported the

distributions mapped 55 years earlier on the basis of morphology (Wright and Wright

1949). Mitochondrial sequence divergence between eastern and western lineages

averaged 3% and reached 4.3%, which is comparable to species-level divergences in

some other members of the genus (Jaeger et al. 2001; Hoffman and Blouin 2004a).

Hoffman and Blouin suggested that the deep divergences between eastern and western

mitochondrial lineages may indicate that what is currently known as the Northern

Leopard Frog in fact consists of two cryptic species, one in eastern North America and

one in western North America. However, they did not recommend recognizing eastern

and western clades as separate species based on their results alone, citing examples of

discordance between mitochondrial and nuclear phylogenies, morphology, and behavior.

The possibility of significant substructuring within this species has been made even more

pertinent because of conservation concerns in the western portion of the species' range

(e.g. Clarkson and Rorabaugh 1989; Werner 2003; McAllister 2005; Germaine and Hays

2009; Moriarty 2009). If Northern Leopard Frogs in the west are distinct from those in

the east, it will be important to begin management actions quickly to preserve this lineage. However, three significant gaps in the understanding of this species preclude an informed decision on whether the western populations are indeed genetically distinct from those in the east. First, it is not known where the geographic border or zone of intergradation between eastern and western mitochondrial clades lies. Second, it is not known whether the nuclear genome shows similar phylogenetic patterns to the mitochondrial genome. Third, it is not known whether eastern and western lineages are reproductively isolated.

The data currently available on the geographic distribution of eastern and western mitochondrial clades are limited to two analyses, one across the range of the species (Hoffman and Blouin 2004a) and one across western Canada (Wilson et al. 2008). Collectively, these analyses showed that most populations sampled were either entirely eastern or entirely western in their mitochondrial DNA. One population was found where eastern and western lineages have come into secondary contact, at the west side of James Bay, Ontario (plus one recent introduction of an eastern frog in Arizona). However, Hoffman and Blouin (2004a) had limited samples that were widely separated in the potential area of overlap between the eastern and western mitochondrial clades (Fig. 3-1). The mitochondrial haplotypes of frogs in most of Ontario and all of Iowa and Wisconsin are unknown. Furthermore, and perhaps more importantly, the geographic extent of introgression between these lineages is unknown. The two clades may be separated by a discrete boundary, or they may have a broad zone of introgression. A very narrow zone of introgression would imply strong reproductive isolation between the clades. If introgression is extremely limited or does not exist, the two clades should be considered

separate species. To determine the geographic extent of the mitochondrial clades and to determine whether the clades meet in a gradual cline of intergradation or discrete parapatry, more sampling is needed at a finer geographic scale between the previously sampled localities.

Second, while the degree of introgression of mitochondrial genes is an excellent tool to begin to investigate introgression between these lineages, it is unknown to what degree mitochondrial phylogenies represent the phylogeny of this species. Discordances between cytoplasmic DNA and nuclear DNA are well known from many other species (e.g. Oyler-McCance et al. 1999; Yang and Kenagy 2009; Fontenot et al. 2011; Jacobsen and Omland 2011). Range-wide analysis of several unlinked nuclear markers is needed to determine whether the deep divergences in mitochondrial DNA indicate a significant level of genome-wide divergence, which would support the recognition of these clades as separate subspecies or even species.

Third, the extent to which the two lineages are reproductively isolated is not known. Using only a mitochondrial marker, as Hoffman and Blouin (2004a) did, hybrids cannot be detected. Determining whether eastern and western clades can interbreed requires analysis of nuclear DNA from the area of overlap between eastern and western mitochondrial lineages. Extensive interbreeding over a broad area would indicate a lack of reproductive barriers between the two lineages. If nuclear and mitochondrial origins in the zone of overlap are strongly correlated, or if the zone of overlap is narrow, then the hypothesis that eastern and western lineages are reproductively isolated would be supported.

Our objective was to address these three gaps in knowledge by testing three corresponding hypotheses. First, we tested the hypothesis that eastern and western mitochondrial clades overlap over a narrow geographic distance. Second, we tested the hypothesis that the distribution of nuclear markers is congruent with the previously described distribution of mitochondrial clades. Third, we tested the hypothesis that the origin of mitochondrial DNA of individuals in the zone of overlap is significantly correlated with the population of assignment based on microsatellite data.

Materials and methods

We collected tissue samples from 17-30 individuals from each of 24 populations around the range of the Northern Leopard Frog, for a total of 567 individuals (Table 3-1 and Fig. 3-2). Most samples were collected in 2008-2010, but some were acquired from previous collections, with the oldest samples from 2004. Populations were chosen to represent the majority of the species' range, with additional sampling concentrated in the vicinity of the Mississippi River, an area where eastern and western mitochondrial haplotypes were thought to meet (Hoffman and Blouin 2004a). Tissue samples were collected by clipping the tip of the third toe on the right hind foot directly into a microvial containing 95% ethanol. To minimize the risk of infection, we applied an antibiotic/anesthetic solution to the cut toes before releasing each frog at its point of capture (Green 2001). Surgical instruments were sterilized between each frog to eliminate the risk of sample contamination and to reduce the risk of spreading diseases among frogs. When possible, we collected tissue samples from freshly road-killed specimens rather than live specimens. Samples were then frozen and stored at -80°C.

DNA was extracted from toe clips using a standard chloroform extraction (Müllenbach et al. 1989). The purified DNA was resuspended in a Tris-EDTA buffer (0.1M Tris, 0.1M EDTA, pH 9.0) and stored at -80° C.

We sequenced one mitochondrial gene, to assess the distribution of eastern and western haplotypes in the zone of potential overlap, and four nuclear sequences (including introns and exons) to compare distribution and phylogeny of nuclear genes to the mitochondrial gene. These loci were sequenced for all sampled individuals from populations WAP, TIF, GWM, COP, and OGD, and at least five individuals from each of the remaining populations. Sequence data was obtained for 786 base pairs of subunit 1 of the mitochondrial gene NADH dehydrogenase (ND1) using the primers RpND1F and RpND1R (Hoffman and Blouin 2004a; Wilson et al. 2008). Each 25 mL polymerase chain reaction (PCR) contained 0.4 µmol/L of each primer, 150 µmol/L dNTPs, 1× standard PCR buffer (including 1.5 mmol/L $MgCl_2$), 2.5 units of Taq polymerase, and ~50 ng of genomic DNA. PCR conditions consisted of 5 minutes of initial denaturation at 95° C; followed by 35 cycles of 94° C for 60 seconds, 54° C for 60 seconds, and 72° C for 90 seconds; followed by a 5 minute final extension at 72° C. Nuclear sequences included segments of two different exons of the rhodopsin gene (283 bp of Rhod1 and 137 bp of Rhod4), one 507 bp segment of the tyrosinase gene (Tyr), and one 199 bp segment of β-fibrinogen intron 7 (FIB; Prychitko and Moore 1997; Bossuyt and Milinkovitch 2000; Di Candia and Routman 2007). PCR conditions were identical to those for amplifying ND1 sequences with the following exceptions. Taq was reduced to 1.25 units for all nuclear loci. For FIB, final $MgCl_2$ concentration was increased to 2.25 mmol/L. Temperature conditions were also identical to those used for ND1 except that

Rhod1 and Rhod4 started with a 10-minute initial denaturation, and their extension

times were 60 seconds. Annealing temperatures were 59° C for Rhod1, 57° C for Rhod4,

and 58° C for Tyr and FIB. PCR products were visualized on a 0.7% agarose gel to

check for product quantity and size. PCR products were purified with QIAquick PCR

purification kit (Qiagen) and sequenced with BigDye chemistry (Applied Biosystems) on

an ABI 3730 sequencer. Sequences were edited and aligned with SeqMan II software.

Sequences were generated in the forward direction first and in the reverse direction if the

sequence could not be confidently read throughout the entire amplicon in the forward

direction alone.

Heterozygotes could be identified directly from overlapping peaks in sequencing

traces, but haplotypes could not be determined directly in individuals that were

heterozygous at more than one base pair. To assign phase (haplotypes association) for

these heterozygous sites, we used the program PHASE (Stephens and Donnelly 2003;

Flot 2010).

TCS version 1.21 was used to identify unique haplotypes in the dataset and to

examine the relationships among those haplotypes using statistical parsimony (Clement et

al. 2000). We also examined the relationships among haplotypes using maximum

likelihood and Bayesian methods. Maximum likelihood phylogenies were constructed

with RAxML 7.2.8 (Stamatakis 2006; Stamatakis et al. 2008) as implemented in CIPRES

with default settings (Miller et al. 2010). Bayesian phylogenies were constructed using

MrBayes, sampling at least 1,000,000 generations (or adding 1,000,000 additional

generations until the standard deviation of split values was less than 0.01) at every 1000th

generation, with nst = 6 and rates with an inverse gamma distribution, and discarding

the first 25% of samples as burn-in.

To further test for geographic patterns in nuclear DNA and to test for evidence of

interbreeding in the zone of introgression, we amplified seven microsatellite loci: Rpi100,

Rpi101, Rpi102, Rpi104, Rpi107, and Rpi108 (Hoffman et al. 2003), and RP193

(Hoffman and Blouin 2004b). PCR conditions were 2 minutes of initial denaturation at

95° C, followed by 30 cycles of the following steps: 95° C for 30 seconds, annealing

temperature for 30 seconds, and 72° C for 1 minute; followed by a 10 minute final

extension at 72° C. Locus-specific annealing temperatures were as follows: Rpi100: 52°

C, Rpi101: 62° C, Rpi102: 50° C, Rpi103: 55°C, Rpi104: 56° C, Rpi107: 52° C, Rpi108:

52° C, and RP193: 56° C. The PCR product was visualized on a 0.7% agarose gel to

check for product quantity and size. PCR products were then analyzed on an ABI 3100

or 3730 sequencer. To search for clusters of similar individuals based on microsatellite

allele frequencies, we used the program STRUCTURE version 2.3 (Pritchard et al. 2000),

following the recommendations for interpreting results from Evanno et al. (2005). After

determining the optimum number of groups using STRUCTURE, we then plotted the

spatial distribution of these groups using GENELAND (Guillot et al. 2005). To test the

significance of structuring both between populations and between larger groups identified

by STRUCTURE, we used AMOVA, implemented in GENALEX version 6.4 (Peakall

and Smouse 2006). To test whether genetic distance between populations was

significantly related to their geographic distance, we used a Mantel test, implemented in

GENALEX (Peakall and Smouse 2006). Finally, to test whether genetic differences were

greater between groups than expected due to isolation by distance, we used a partial

Mantel test, implemented in ZT (Bonnet and Van de Peer 2002). Specifically, we used

the partial Mantel test to determine whether genetic distance differed between categories

of population pairs (within east, within west, or across the east-west boundary), after

controlling for geographic distance. For this test, we excluded populations that were

indicated by STRUCTURE to be in the zone of introgression (see results). We used these

varied approaches to help overcome any inherent biases in each individual approach: it is

common for various methods to give different results, and a broad perspective is

necessary to draw correct inferences from analyses such as these, especially when

isolation by distance is evident (Frantz et al. 2009).

 To look for genetic evidence of reproductive barriers, we first selected

populations where several individuals assigned to more than one group of populations

(see results), and then we conducted two separate tests on these populations. First, we

compared probability of assignment to one group among individuals with eastern or

western mitochondrial haplotypes using a Wilcoxon rank-sum test. Strong genetic

isolation would cause the probability of microsatellite-based group assignment to be

highly correlated with mitochondrial haplotype. Second, we performed a Principal

Coordinates Analysis (PCA) on microsatellite allele frequencies among individuals in

these populations. If reproductive isolation were complete, we would expect two discrete

groups to be visible in the PCA. If the two lineages can reproduce but produce infertile

offspring, we would expect two discrete groups representing each lineage with a third

discrete group between them, representing the F1 hybrids. If no reproductive barriers

exist, or if reproductive barriers are present but incomplete, we would expect to see all

individuals in a single cluster in the PCA analysis.

Results

Mitochondrial sequencing

ND1 sequencing revealed two groups of related haplotypes that differed from each other by 2.7% to 4.5% sequence divergence (mean = 3.8%, uncorrected). These two groups did not connect in a 95% parsimony diagram with a connection limit of 12 steps (Fig. 3-3). Within-group divergences ranged from 0.1% to 1% in the eastern group and 0.1% to 0.9% in the western group. These two haplogroups were strongly supported (98% bootstrap support), but had little resolution among haplotypes within each group. These haplogroups corresponded to the eastern and western groups described by Hoffman and Blouin (2004a). Only two of the 24 populations we sampled had both eastern and western haplogroups. These two populations were both on the Mississippi River: WAP on the west side of the river in Iowa (four eastern, 18 western) and TIF on the northeast side of the river in Wisconsin (two eastern, 22 western). All other populations west of the Mississippi River consisted of 100% western haplotypes, and all other populations east of the Mississippi River consisted of 100% eastern haplotypes.

Nuclear sequencing

PHASE was able to assign haplotypes in heterozygotes with varying success. For Rhod1, all haplotypes were assigned with 100% confidence except for one position in one individual that was ambiguous (50% confidence for each of two assignments). For Rhod4, all haplotypes were assigned with 100% confidence. For Tyr, some sites had confidence as low as 58%. For this gene, we used two approaches for phylogeny reconstruction, one including all haplotypes as determined by majority (> 50%) support,

and one including only haplotypes determined with 100% confidence. For FIB, all

haplotypes were assigned with at least 98% confidence.

For Rhod1, we found only three haplotypes (Fig 3-4). One of these was very rare

and differed by only one bp from a second haplotype that was widespread in the western

part of the range; together these were considered a western group. The third haplotype

was found throughout the east, and we considered it an eastern form. Eastern and

western haplotypes differed by 1.1 to 1.4% sequence divergence (mean 1.2%). The

eastern haplotype was well supported (99% bootstrap support) as being separate from the

western haplotypes. Both eastern and western haplotypes were found at four of the 24

populations we sampled. WAP and GWM had mixed Rhod1 haplotypes (WAP: 3

eastern, 37 western; GWM: 3 eastern, 45 western) and were in the area of overlap

between eastern and western lineages according to our mitochondrial sequencing results.

Two additional populations, LAR in eastern Ontario, and HIS in western New York, each

had a single western haplotype (out of 10 for LAR and 12 for HIS).

We detected four haplotypes for Rhod4 (Fig 3-5). Three of these haplotypes were

closely related and prevalent in the west. We considered these a western haplogroup.

The fourth haplotype was generally limited to the east and we considered this the only

representative of the eastern haplogroup. Western haplotypes differed by one to two bp

(0.7 to 1.5%). Western haplotypes differed from eastern haplotypes by 2.2 to 2.9%

(mean 2.7%). The distribution of Rhod4 haplotypes was very similar to that of Rhod1

haplotypes. This was not surprising given that Rhod4 is physically located very near

Rhod1 and therefore low levels of recombination are expected between these sequences.

About 1,700 base pairs separate these genes in the Western Clawed Frog (*Xenopus*

tropicalis; xenbase.org), and the distance is presumably similar in the Northern Leopard Frog. The area of geographic overlap between eastern and western haplogroups was nearly identical to that described for Rhod1. Only one individual had a Rhod4 haplogroup that did not match its Rhod1 haplogroup (n = 312), as expected for two sequences that are physically very close in the genome. The only difference from the distribution of Rhod1 was one individual at LAR that was homozygous at Rhod1 and heterozygous at Rhod4.

Tyr was much more variable than any of the other sequences studied. Haplotypes found in the east generally grouped together but with very weak bootstrap support (8–39 %, depending on the haplotype), therefore we did not have the power required to detect a geographic difference had there been one. Because we could not assign haplotypes to groups with confidence, we were unable to spatially map their distribution.

We used FIB because previous authors have shown it to be informative for phylogenetic questions in the same genus (Di Candia and Routman 2007). However, contrary to these authors' results, we did not find that the variation in length of this gene was indicative of species. The insertion/deletion (indel) Di Candia and Routman (2007) described as being diagnostic of Plains Leopard Frogs (*Rana blairi*) also was widespread among western Northern Leopard Frogs, which greatly complicated the analysis of heterozygous individuals. There were at least two different indels in the sequences we amplified from Northern Leopard Frogs. Because PHASE is not well suited to determining phase in the presence of indels, we excluded the part of the gene with the indels, leaving a much shorter segment for analysis than previous authors have used (199 bp versus up to 615 bp). Despite this short read, we found 10 different haplotypes among

our FIB sequences (Fig 3-6). These grouped into four haplogroups, but there was no

clear distinction between east and west in this locus. One haplogroup (blue in Fig. 3-6)

was the only one present in the easternmost populations. Defining this group as eastern

and the remainder as western, there was as much variation among western haplotypes as

there was between eastern and western (mean 1.6% divergence). This eastern haplogroup

was found as far west as central Nebraska, northern Minnesota, and western Ontario (Fig.

3-6). This was further west than the western limit of eastern haplotypes in ND1, Rhod1,

and Rhod4. Conversely, western FIB haplotypes were found as far east as eastern

Ontario and central New York. This finding is similar to the eastern limit of eastern

haplotypes of Rhod1 and Rhod4.

Microsatellite loci

Seven microsatellite loci were used to assess the phylogeography of the nuclear

genome by simultaneously using a greater number of loci. Using a Bayesian clustering

algorithm, STRUCTURE, individuals optimally divided into two groups (Fig. 3-7). These

groups corresponded well with the eastern and western clades identified by mitochondrial

sequencing. Only two populations (WAP and TIF, both on the Mississippi River)

showed mixed assignments of eastern and western individuals. These are the same two

populations that contained eastern and western mitochondrial haplotypes. Ninety-one

percent of the remaining individuals assigned to eastern or western groups with >90%

confidence. Assignment of individuals to two groups by GENELAND aligned very

closely with results from mitochondrial, Rhod1, and Rhod4 sequences (Fig 3-8).

We used AMOVA as a secondary method to test the significance of genetic

differences between the two groups that were indicated by STRUCTURE and to test for

differences between the pre-defined populations from which we sampled. There were

significant differences in allele frequencies among populations (P = 0.001). All pairwise

p-values (not corrected for multiple tests) were less than 0.001, the limit of precision for

this test. AMOVA also confirmed the presence of a significant difference between the

eastern and western groups that had been identified by STRUCTURE (P = 0.001).

Isolation by distance was significant across the range (Fig 3-9). Genetic distance (Φ_{PTP})

between population pairs was significantly related to geographic distance (Mantel test, R^2

= 0.263, P < 0.001). Genetic distance between populations was greater within the west

than between west and east (partial Mantel test, r = -0.26, P = 0.001) or within the east

(partial Mantel test, r = 0.62, P = 0.0001). PCA supported the hypothesis that

populations close in geographic distance are also generally close in genetic distance.

PCA showed a horseshoe shape, which is a common pattern when displaying data across

a linear gradient (Fig 3-10). In this case, the linear gradient was from eastern to western

populations, which formed distinct clusters but which were not widely separated from

one another, and which were joined in principal coordinate space by populations with

mixed assignments (WAP and TIF, on the Mississippi River).

Hybridization within individuals

To test for genetic evidence of robust reproductive isolation between eastern and

western lineages in these two populations, we compared the probability of assignment to

the eastern group, as computed by STRUCTURE, between two groups of individuals

from the area of overlap (TIF and WAP), with eastern versus western mitochondrial haplotypes. Probability of assignment to eastern or western groups on the basis of microsatellite alleles was not related to mitochondrial haplotype (Wilcoxon Rank-Sum Test, $Z = 0.19$, one-tailed $P = 0.43$), indicating that individuals in these populations had a mix of eastern and western ancestry. Secondly, we conducted a PCA of microsatellite alleles from individuals in these two populations. PCA showed a single cluster of individuals, which is expected in the absence of a robust reproductive barrier (Fig. 3-11).

Discussion

Previous conclusions about subspecific differentiation in Northern Leopard Frogs have ranged from supporting a phylogenetic split within the species on the basis of mitochondrial DNA (Hoffman and Blouin 2004a), to showing no difference between putative subspecies on the basis of allozymes and call characters (Dunlap and Platz 1981). The results presented here provide evidence that Northern Leopard Frogs include two lineages with significant genetic differences, evident in both mitochondrial and nuclear genomes, although the strength of this signal varies among markers used. Nuclear sequencing showed some deep divergences with limited introgression between them, but some loci (FIB and Tyr) lacked evidence of genetic differentiation associated with geography. In contrast, mitochondrial sequencing showed deep divergence between eastern and western clades with very little geographic overlap between them. Significant differentiation was also shown by the combination of seven microsatellite loci, with a narrow range of overlap that coincided closely with the patterns shown by mitochondrial sequencing.

For two cryptic species that are completely reproductively isolated, a pattern that looks like introgression can be the result of partial geographic overlap between the species. Our individual-level analysis of populations WAP and TIF confirm that a robust barrier to reproduction does not exist between these two lineages, because PCA did not identify two isolated groups within these populations and because mitochondrial haplotype was not significantly correlated with group assignment based on microsatellites. This does not exclude the possibility of reduced hybrid fitness or partial reproductive isolation—experimental matings would be required to address these possibilities—but we can conclude from these data that any reproductive barriers are at least somewhat permeable.

Because our results differ between loci and marker types (sequencing vs. microsatellite), it is important to take a holistic approach to assessing what these data reveal about subdivision within this species (Sites and Marshall 2003, 2004). The extent of introgression that we found ranged from very narrow for mitochondrial sequences and microsatellite allele frequencies, to more extensive for nuclear sequences. There are at least four possible explanations for this apparent discordance. First, our microsatellite analyses simultaneously incorporate data from seven different loci, whereas sequencing analyses are done on a locus-by-locus basis, and are more susceptible to locus-specific stochastic effects of gene flow and genetic drift. Gene trees are not equivalent to species trees, and any one gene is likely to have a phylogeny that does not precisely match the phylogeny of the organism that contains it, due to these stochastic factors.

Second, our nuclear and mitochondrial sequences are also more likely to be subject to the effects of selection than microsatellite loci, because of their location in or

proximity to coding regions. Selection acting on genes can affect the geographic

distribution of alleles in ways that are not typical of the rest of the genome. For example,

in intergrading subspecies of house mice, the extent to which alleles are exchanged

between subspecies depends on the selective advantage of the allele being exchanged

(Selander et al. 1969). This potential may explain our results for fibrinogen, which

showed the broadest introgression of all nuclear loci we sequenced. Fibrinogen is

important in the clotting of blood after an injury. A significant heterozygote advantage

can promote the spread of genes across a hybrid zone (e.g. Fitzpatrick and Shaffer 2007).

Significant heterosis has been demonstrated for the fibrinogen gene in cattle, where

heterozygotes have higher plasma levels of fibrinogen than inbred cattle (Qiu et al. 2007).

If a similar heterozygote advantage exists in frogs, this could explain the greater spread

of fibrinogen genes across the zone of contact than expected on the basis of microsatellite

and mitochondrial data.

A third possible explanation for the wide distribution of some of our nuclear

sequences across the zone of contact is that it results from incomplete lineage sorting

rather than introgression. Because each individual has two copies of nuclear DNA, and

mitochondrial DNA is generally single-copy and maternally inherited, the effective

population size for mitochondrial DNA is only one-fourth that of nuclear DNA. This

smaller effective population size means that mitochondrial alleles from pre-Pleistocene

contact are more likely to be lost due to drift than nuclear alleles. Compounding this

effect is the fact that substitution rate for mitochondrial DNA is approximately four to six

times faster than for nuclear DNA (Johnson and Clayton 2000; Weibel and Moore 2002),

so new mitochondrial haplotypes are more likely to emerge in a period of isolation. The

strongest evidence for introgression is the geographic distribution of haplogroups. If two lineages were present in a geographic area because they had not completely sorted, both lineages would be expected to be randomly distributed within the range of the clade. Strong geographic trends in allele frequency are evidence of introgression, and can be discerned in our results for ND1, Rhod1, Rhod4, and the seven microsatellites. In contrast, FIB and Tyr do not show distinct clades associated with geography, and are more likely to be explained by selection or incomplete lineage sorting between recently diverged lineages.

Finally, evidence from a well-studied hybrid zone in mice (*Mus musculus* and *M. domesticus*) indicates that mitochondrial genes are less likely than nuclear genes to introgress because of the lack of recombination in the mitochondrial genome (reviewed in Sage et al. 1993). Individual nuclear genes are more likely to introgress across a hybrid zone because they have only a small effect on coadapted gene complexes, but because mitochondrial DNA is inherited as an entire cytoplasmic genome, the negative effects of a mitochondrial genome mismatching with a nuclear genome are more dramatic and detrimental. Thus, cytoplasmic DNA typically shows a steeper differentiation across a hybrid zone than does nuclear DNA.

Together these factors make it apparent that, despite introgression or incomplete sorting in some nuclear sequences, there are significant genetic differences between eastern and western Northern Leopard Frogs. In light of these genetic differences, it may be worth reevaluating whether there are also phenotypic (morphological, immunologic, metabolic, or behavioral) differences between the east and west in this species. Western populations (eastern Great Plains to Sierra Nevada) were initially considered a

subspecies, *Rana pipiens brachycephala*, separate from nominate *Rana pipiens pipiens*

on the basis of a variety of morphological features (Cope 1889). The western subspecies

was elevated to species status by Kauffeld (1937) on the basis of Cope's morphological

features and a few additional features. Kauffeld (1937) extended the range of

brachycephala as far as the eastern coast of North America, south to parts of

Pennsylvania. Using this definition, Moore (1944) failed to find any consistent

morphological differences between the species of leopard frogs. He thus considered

brachycephala to be a synonym of *pipiens*. Wright and Wright (1949) agreed with Cope

in considering *brachycephala* to be a valid subspecies and argued that its distribution was

limited to the west; they considered populations in the northeastern United States and

southeastern Canada to be *Rana p. pipiens*. They mapped the ranges of *Rana p. pipiens*

and *Rana p. brachycephala* with parapatric distributions that coincide very closely with

the genetic discontinuities noted here. Dunlap and Platz (1981) attempted to address

reported differences between *R. p. pipiens* and *R. p. brachycephala* by studying allozyme

variation and two acoustic properties of calls across a transect from Idaho to Wisconsin.

They found no consistent differences in allozymes (n = 85) or calls (n = 10) and

concluded that *Rana pipiens* should be considered monotypic. However, calls of this

species are among the most complex of any temperate anuran (Larson 2004), and analysis

of only two acoustic parameters from ten individuals may be insufficient to detect

regional differences in calling behavior. Furthermore, allozyme analyses may be

insufficient because there are several cases in other species where analysis of allozymes

failed to detect genetic structuring that was later revealed by analysis of DNA sequences

(Avise 2000). For example, allozymes did not significantly differ between Japanese and

Korean Pond Frogs (*Rana nigromaculata*), but mitochondrial DNA showed that

Japanese *R. nigromaculata* are more closely related to *R. plancyi chosenica* than to

Korean *R. nigromaculata* (Kim et al. 2004). For this reason, we are not surprised to see

that our analysis, based on a combination of mitochondrial sequencing, nuclear

sequencing, and nuclear microsatellites, revealed more significant substructuring than

previous work with this species based on allozymes. Perhaps because of the lack of

differentiation in allozyme frequencies, the morphological features of Cope and Kauffeld

have not been critically evaluated in the context of a strictly western *R. p. brachycephala*.

Changes in morphology have been noted across the zone of introgression in northern

Canada (Schueler 1973), and an analysis of morphological features generally separated

eastern and western frogs, with frogs from the Great Lakes region intermediate (Fig. 10

in Schueler 1982). Given the genetic differences reported here and by Hoffman and

Blouin (2004a), and their correlation with previous mapped distributions (Wright and

Wright 1949) and, roughly, with spotting patterns (Schueler 1982), additional analysis of

phenotypes is warranted. It seems likely that such work will reveal morphological or

acoustic traits helpful in distinguishing these two genetic lineages, perhaps justifying the

resurrection of *Rana pipiens brachycephala*.

These findings contribute to the growing understanding of phylogeography of

North American anurans, and of North American species in general. The Mississippi

River occurs as a recurring biogeographical barrier among clades in anurans. For

example, both American Bullfrogs (Ranidae: *Rana catesbeiana*) and Spring Peepers

(Hylidae: *Pseudacris crucifer*) show evidence of intraspecific clades having secondary

contact after the Pleistocene around the Mississippi River (Austin et al. 2004b). Species-

level differentiation among the chorus frogs (*Pseudacris* spp.) also shows east-west

divergences, separated by the Mississippi River (Moriarty and Cannatella 2004). Indeed,

the Mississippi is an important barrier in many taxa (reviewed in Soltis et al. 2006), for

example, turtles (Walker et al. 1998), shrews (Brant and Ortí 2003), and trees (Al-

Rabab'ah and Williams 2002). It appears that the same barrier is important, albeit

partially permeable, in the Northern Leopard Frog, separating two unique genetic

lineages with independent evolutionary histories.

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Table 3-1: Sources of samples. Some location names reference nearby (< 5 km) locations. Longitude and Latitude are means of all samples, given in decimal degrees, WGS84 projection. NF = National Forest, NWR = National Wildlife Reserve, RM = Rural Municipality, SF = State Forest, SRA = State Recreation Area, SWA = State Wildlife Area, WMA = Wildlife Management Area. Populations are arranged from west to east.

Abbr.	Location	Longitude	Latitude	N
POT	Potholes Lakes, Grant Co., WA	-119.371	47.088	24
TRU	Truxton Wash, Mojave Co., AZ	-113.640	35.424	28
CHO	Chocolate Pond, Bow City, AB	-112.268	50.432	17
OUR	Ouray NWR, Uintah Co., UT	-109.626	40.144	24
WCO	West Coyote Wash, San Juan Co., UT	-109.296	38.289	22
SWE	Sweetwater River, Carbon Co., WY	-107.289	42.419	24
CUS	Custer NF, Carter Co., MT	-104.210	45.702	24
HOR	Horsehead Creek, Fall River Co., SD	-103.299	43.121	20
NPL	North Platte River, Keystone, Keith Co., NE	-101.631	41.219	24
WSL	West Shoal Lake, Shoal Lake RM, MB	-97.595	50.456	30
TWO	Two Rivers SRA, Douglas Co., NE	-96.343	41.215	24
OGD	Harrier Marsh WMA, Boone Co., IA	-94.017	42.023	20
MOO	Moose-Willow WMA, Aitkin Co., MN	-93.537	46.952	24
SLA	Sandy Lake First Nation, ON	-93.357	53.065	24
TIF	Tiffany SWA, Buffalo Co., WI	-92.018	44.576	24

WAP	Mill Race Flats WMA, Louisa Co., IA	-91.189	41.192	22
GWM	Mead WMA, Marathon Co., WI	-89.858	44.700	24
COP	Copper Country SF, Houghton Co., MI	-88.525	46.921	24
KRW	Kentucky River WMA, Henry Co., KY	-84.914	38.426	24
OPI	Opinaca River, Route-du-Baie-James, QC	-77.243	52.395	24
HIS	Howland's Island WMA, Cayuga Co., NY	-76.697	43.078	24
LAR	Larose Forest, Co. Prescott and Russell, ON	-75.204	45.396	24
SCO	Scovil, St. John River, NB	-66.104	45.775	24
JON	Jones Pond, Cape Tormentine, NB	-63.822	46.130	24
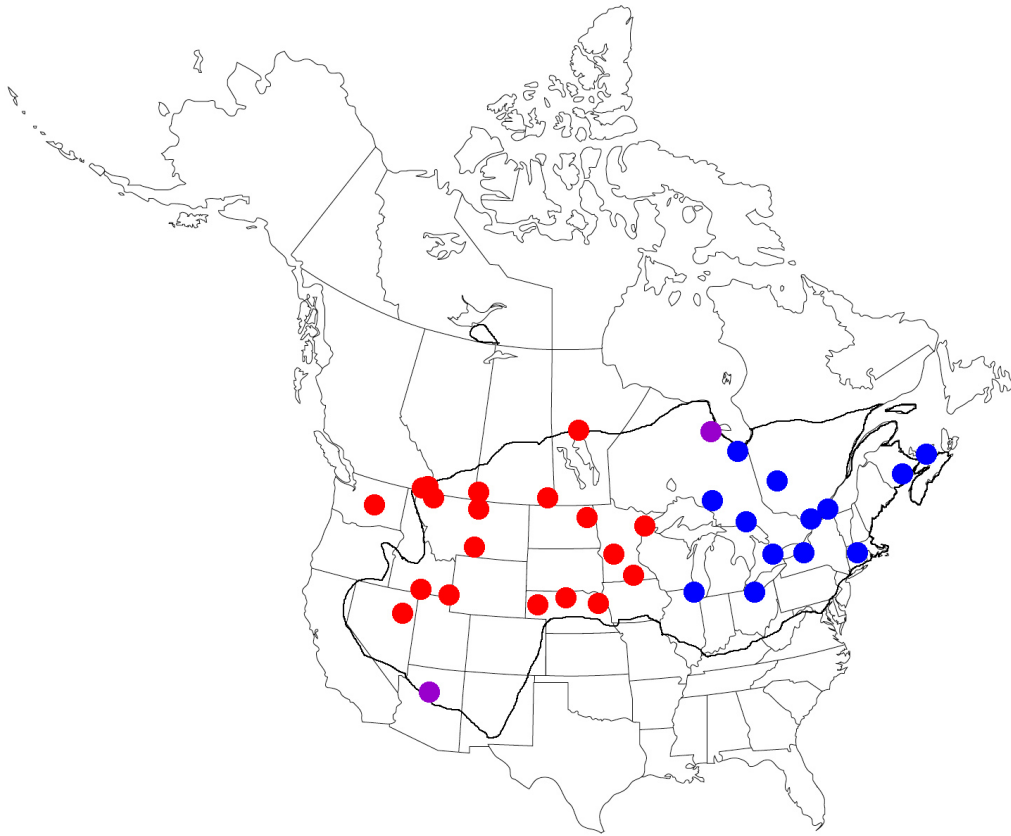
Fig. 3-1: Mitochondrial haplotype groups from Hoffman and Blouin (2004a). Two major haplotype groups are indicated by two colors, red and blue. Populations indicated in purple contained both haplotype groups. The population in Arizona was reported to contain the eastern group as a result of recent introduction. Sample sizes range from 5 to 15 individuals per population.
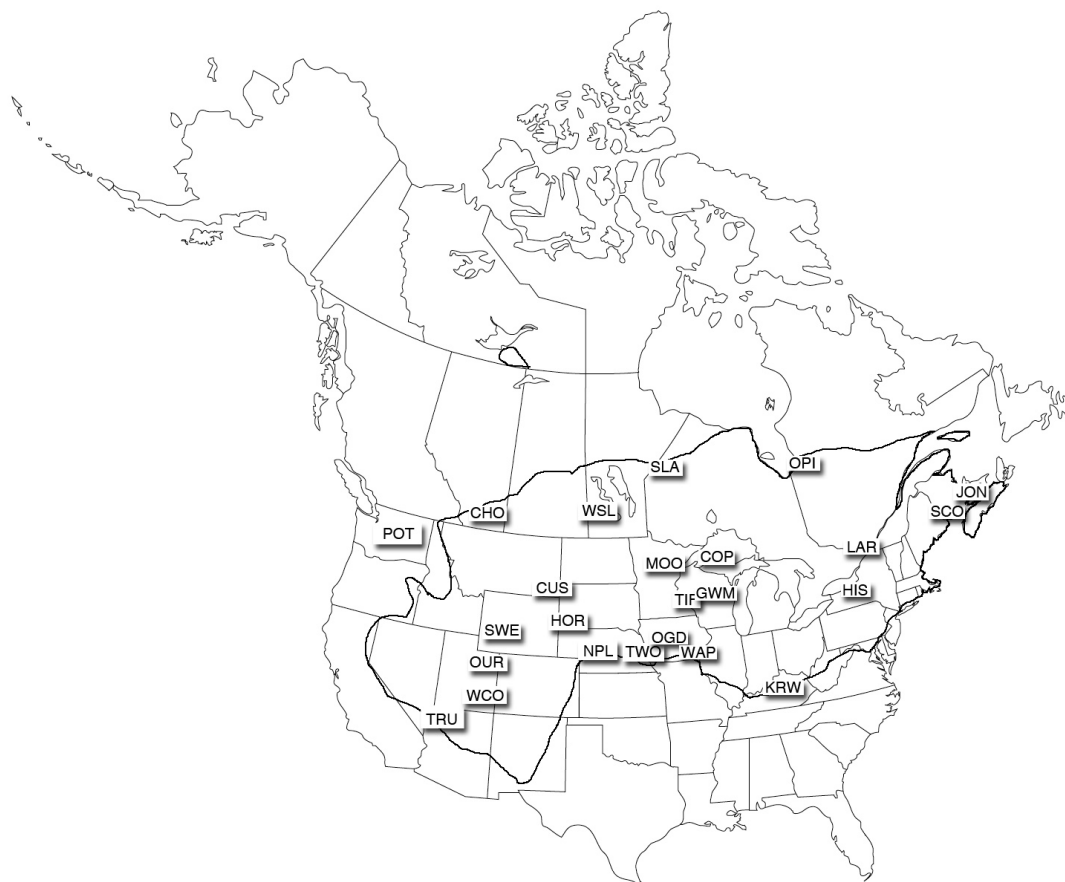
Fig. 3-2: Map of populations sampled in this study. See Table 3-1 for sample sizes, geographic coordinates, and descriptions of localities.
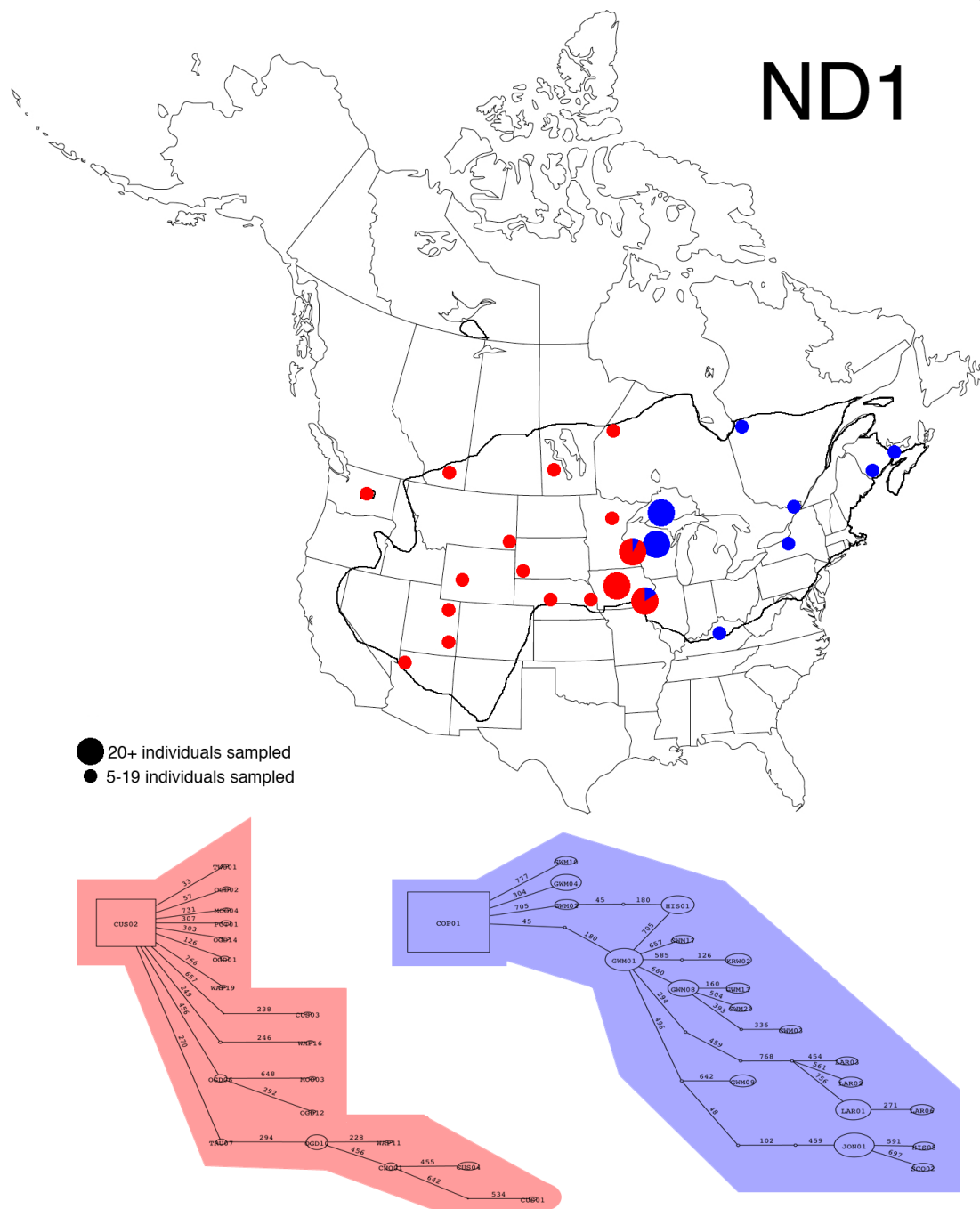
Fig. 3-3: ND1 haplogroups and their distribution. Two parsimony diagrams did not join (95%, connection limit 12). One of these haplogroups was limited to the east, and the other to the west. Black line marks approximate range of the Northern Leopard Frog.
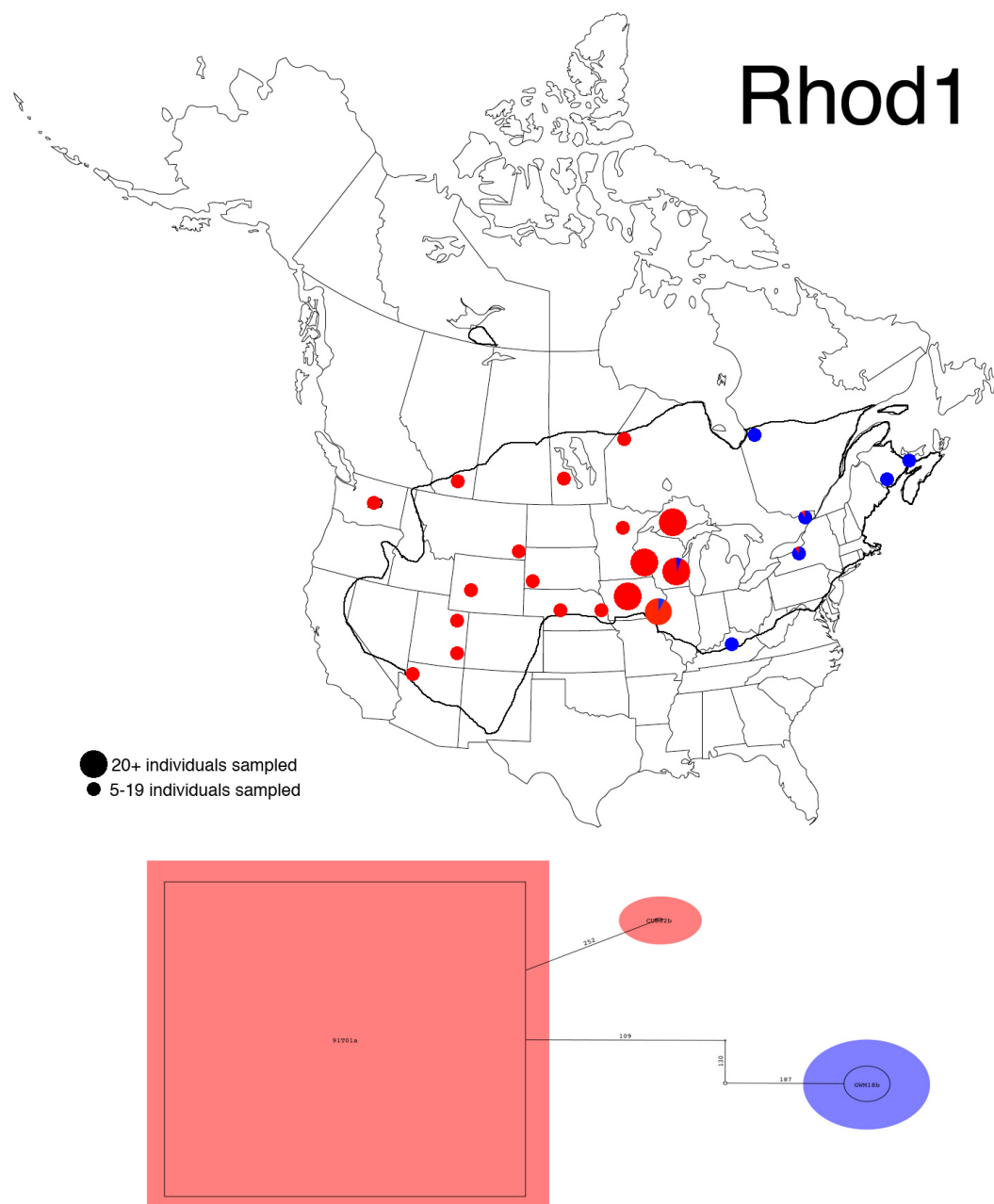
Fig. 3-4: Rhod1 haplogroups and their distribution.
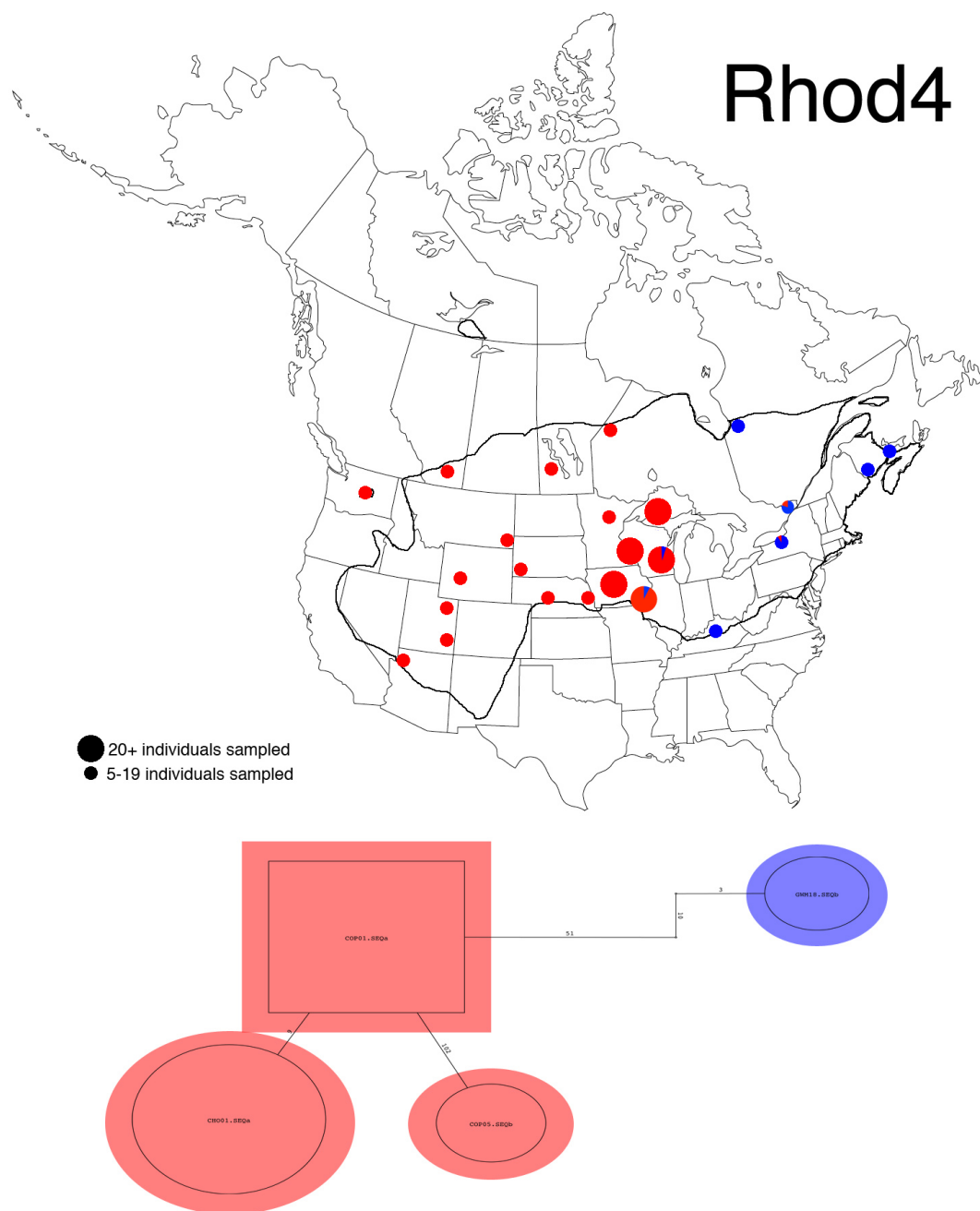
Fig. 3-5: Rhod4 haplogroups and their distribution.
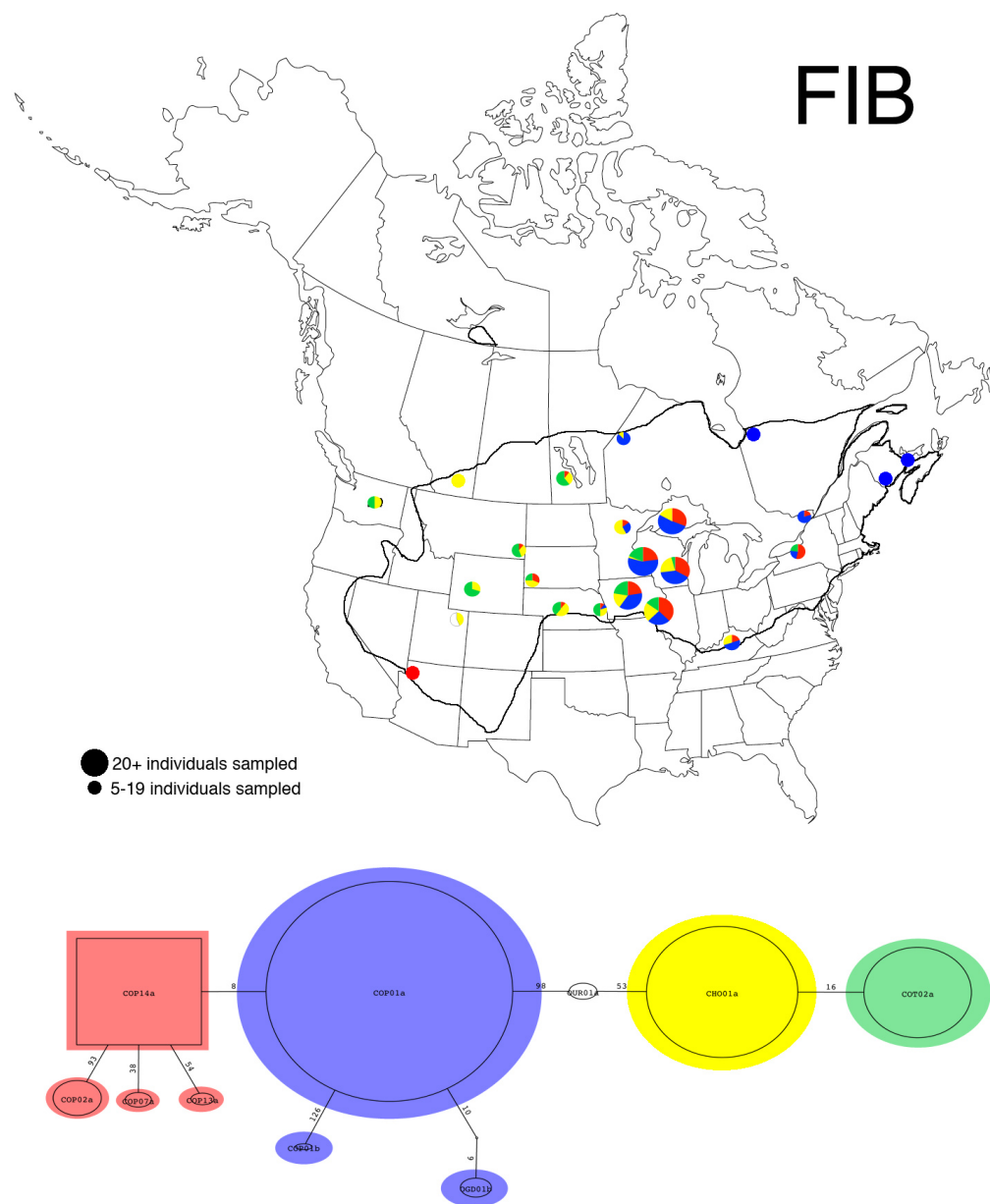
Fig. 3-6: FIB haplogroups and their distribution. Five samples at WCO did not amplify.
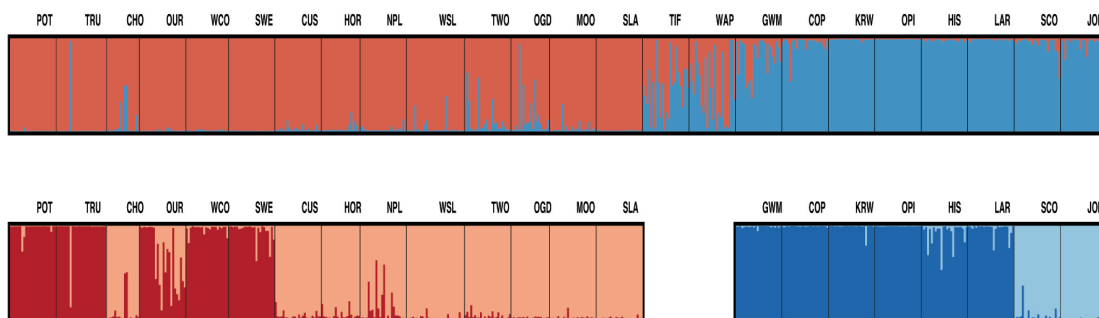
Fig. 3-7: Probability of assignment of individuals, represented by colors of vertical bars, to two groups by STRUCTURE. Populations are arranged by geographic proximity, generally from west (left) to east (right). Top bar is a single run of all individuals; Lower bars represent additional runs on subsets of the whole dataset. Individuals tend to assign to eastern or western groups, with mixed assignments in the two populations on the Mississippi River, WAP and TIF. Within the western group, populations sort into a northeast and southwest group. Within the eastern group, two populations from New Brunswick, SCO and JON, are distinct from the others. See Fig. 3-2 for geographic distribution of populations.
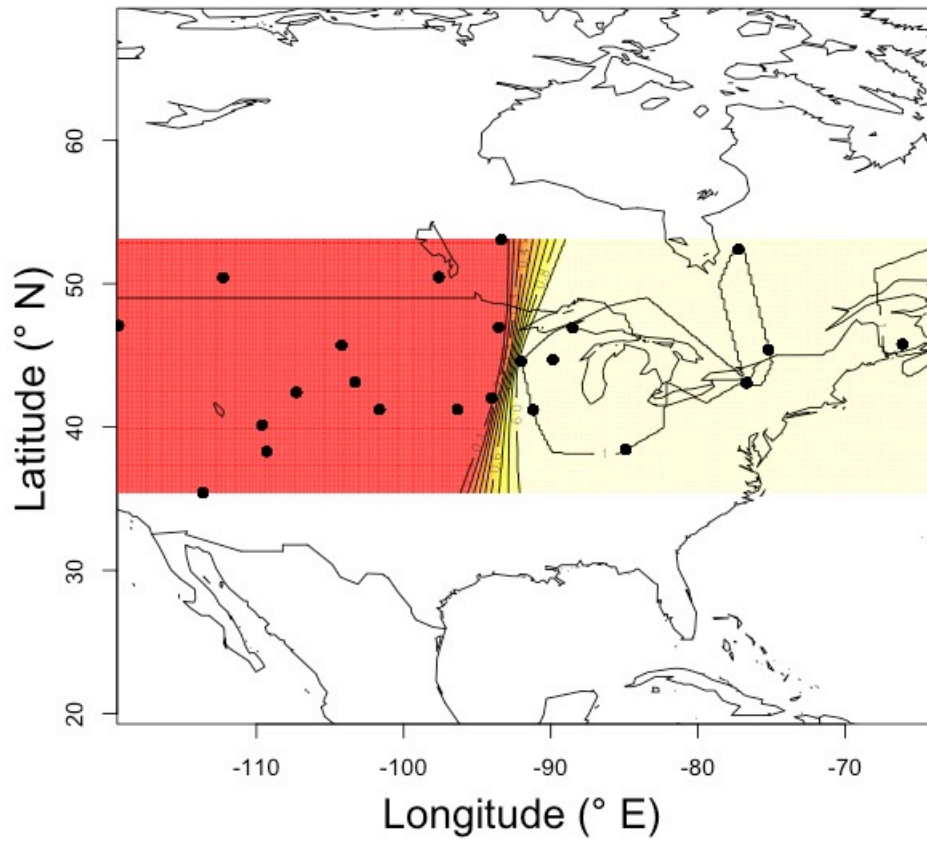
Fig. 3-8: Posterior probability of assignment of microsatellite allele frequencies to an eastern group, calculated by GENELAND. Black dots represent sampled populations. Probability of assignment to a western group is (1-probability of assignment to eastern group).
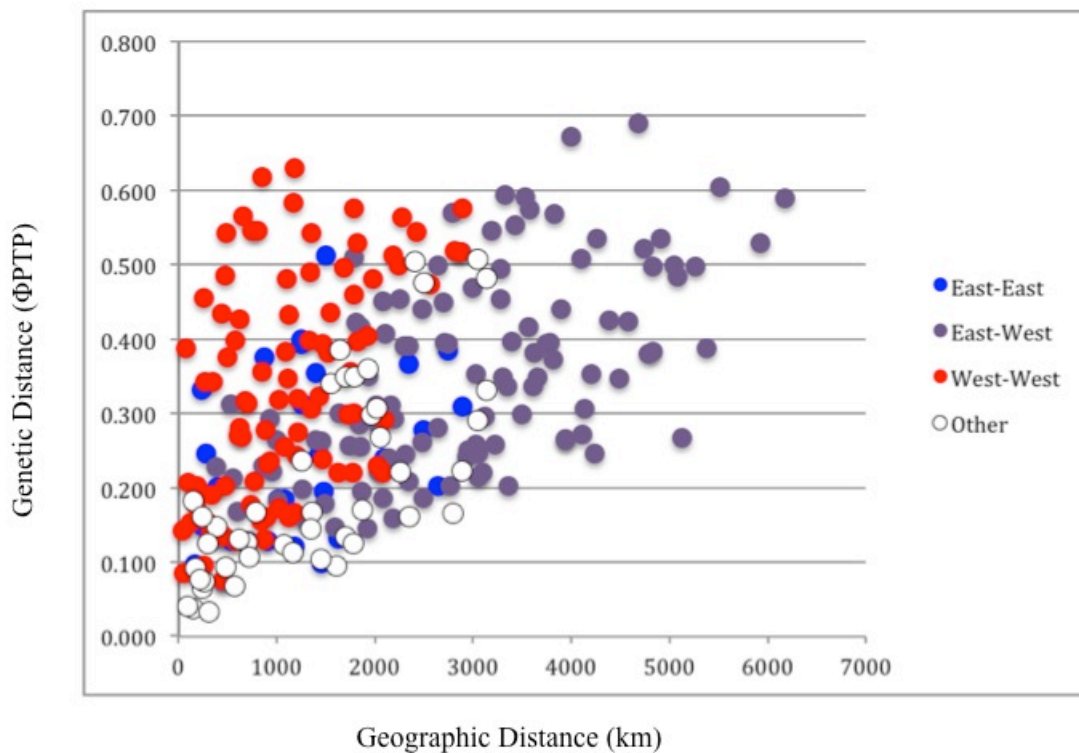
Fig. 3-9: Isolation by distance. Each point shows the genetic distance (Φ_{PTP}) between two populations as a function of straight-line geographic distance (km), calculated by GENALEX. Populations were defined as east or west according to Fig. 3-7, any pair including the intermediate populations was considered "other". Across categories, genetic distance is significantly related to geographic distance.
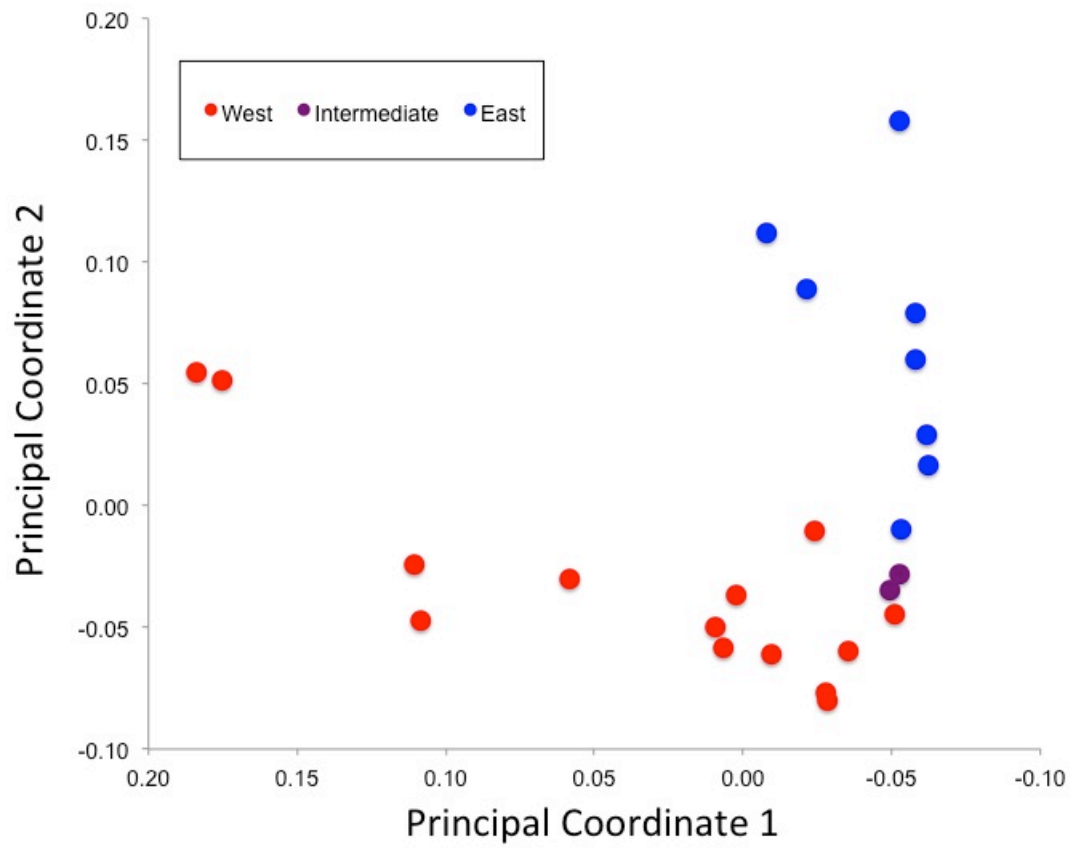
Fig. 3-10: Principal coordinates analysis of 24 populations. Populations are color coded according to assignments from Fig. 3-7.
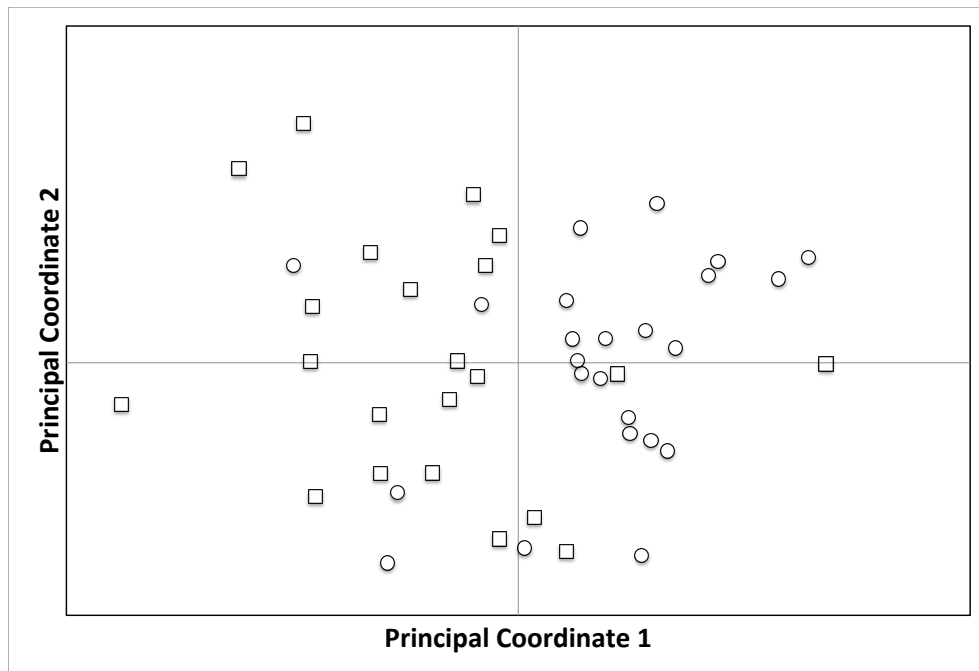
Fig. 3-11: Principal coordinates analysis of microsatellite data from 46 individuals in the intermediate populations, TIF and WAP, on the Mississippi River. Circles are individuals from TIF and squares are individuals from WAP. The lack of two distinct groups is evidence that in the area of overlap between eastern and western clades, at least some introgression has occurred.

CHAPTER 4

GENETIC FORENSICS REVEAL TRANSLOCATION OF NORTHERN LEOPARD

FROGS (*RANA PIPIENS*) ACROSS PHYLOGEOGRAPHIC BOUNDARIES[5,6]

Abstract

Isolation of populations by habitat fragmentation threatens the persistence of many species. In the southwest United States, amphibian habitat is naturally fragmented by broad stretches of dry inhospitable habitat. Northern Leopard Frogs have experienced dramatic population declines in this region, and these recent declines are suspected to have exacerbated the isolation of the remaining populations. We analyzed genetic diversity within and genetic divergence among populations of Northern Leopard Frog throughout Arizona to assess the current levels of isolation among these populations. We also analyzed mitochondrial DNA to place these populations into a larger phylogenetic framework and to determine whether any populations contained genetic material not native to the region. In general, we found a high level of genetic divergence between populations, and low genetic diversity in the isolated populations; however, one complex of populations around the Stoneman Lake area had high genetic diversity and relatively high gene flow among populations. This complex of populations also contained a mitochondrial haplotype that was from eastern frogs, probably representing the introduction of released pets or laboratory animals. These eastern haplotypes were well integrated into this complex of ponds, and probably contributed to the high genetic

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[5] This chapter is co-authored by Ryan P. O'Donnell, Charles Drost, and Karen E. Mock.
[6] This chapter was included as part of a peer-reviewed U.S. Geological Survey Open-File Report, number 2011-1186.

diversity in this area. Genetic diversity in the outlying populations is low and shows

signs of recent bottlenecks, but supplementing genetic diversity in these native

populations with artificial gene flow from the Stoneman Lake area would only be

recommended in extreme situations where no alternatives exist, as it would be ideal to

retain as much genetic integrity of these remaining populations as possible.

Introduction

The loss of connectivity among habitat patches and the resulting fragmentation of

natural populations are important concerns for the long-term preservation of natural

populations. Metapopulation studies of some anuran amphibians have demonstrated a

loss of connectivity among formerly extensive populations and have noted the potential

contributing role of such fragmentation to amphibian declines (Mann et al. 1991; Sjögren

1991; Lehtinen et al. 1999). A variety of causes have been implicated in fragmentation

of amphibian habitat, including clearing of forest lands (Gibbs 1998), road-building (Vos

and Chardon 1998), and introduction of non-native species, particularly predatory fishes

(Bradford et al. 1993). Isolation of subpopulations within habitat fragments, and gradual

loss of these subpopulations over time, may be an important reason for regional declines

(e.g. in *Rana muscosa*; Bradford et al. 1993).

In the southwestern United States, there is considerable natural fragmentation of

amphibian populations by broad stretches of hot, dry, inhospitable habitat separating

seasonal and permanent wetlands. For this reason, the riparian areas along rivers and

streams are vital habitat for many amphibians in this arid country, and provide extensive

corridors linking populations. Over the last 150 years, however, there has been

considerable degradation and loss of such habitats (Dahl 1990; Bogan et al. 1998),

with consequent declines of species that depend on them. Some studies have assessed

direct effects of such habitat loss and degradation on species persistence (e.g. Krueper

1993), but few have considered secondary effects such as isolation of tributary streams,

side canyons, and headwater springs, and the resulting population fragmentation this may

cause. Because of limited dispersal abilities and susceptibility to desiccation in arid

habitats away from water, amphibians appear to be particularly vulnerable to this kind of

population fragmentation.

Northern Leopard Frogs (*Rana pipiens*) formerly occurred in a variety of stream,

pond, and marsh habitats throughout the highland areas of northern Arizona and

neighboring states. Over the last 40 years, however, leopard frogs have experienced

marked declines throughout the southwestern United States (Corn and Fogleman 1984;

Clarkson and Rorabaugh 1989; Sredl 1998). Northern Leopard Frogs are currently listed

as species of conservation concern by a variety of state and federal agencies, including

the Arizona Game and Fish Department ("Species of Special Concern"), the State of

Colorado ("Special Concern Species"), the U.S, Forest Service ("Sensitive," Regions 2

and 3 (Colorado, New Mexico, and Arizona), and the Navajo Nation ("Threatened").

Northern Leopard Frogs were recently considered for listing as a federally threatened

species in the western portion of their range in the United States (Moriarty 2009).

Extant Northern Leopard Frog populations in Arizona are limited to a few isolated

areas in the northern part of the state. There are likely to be multiple levels of genetic

differentiation and isolation among populations within these different areas, which will

be important in assessing conservation and management needs. For these populations, a

major concern is that most or all of them are now effectively isolated from each other. Analysis of genetic data from the extant populations in Arizona will allow assessment of patterns of genetic diversity, isolation, and recent population bottlenecks in many of the populations of Northern Leopard Frogs in Arizona. This analysis may be crucial in understanding likely population trends and long-term viability of populations throughout the region as well as informing management decisions about translocations and habitat restoration efforts.

A primary concern in the management of Northern Leopard Frogs in Arizona is the planning of translocations to reestablish the species where it has been recently extirpated. The objective of this study is to determine which populations would be suitable sources for translocations. Because of its relatively high genetic diversity and large population sizes (Kimberling et al. 1996), the Stoneman Lake area is a likely source of individuals for translocations. However, a recent range-wide genetic study on the Northern Leopard Frog revealed at least one individual from an eastern clade of the species present in this area (Hoffman and Blouin 2004a). Because of deep divergences between eastern and western clades, and the potential for outbreeding depression resulting from introgression between the two clades, we sought to assess the prevalence and distribution of the eastern mitochondrial haplotype across Arizona before recommending a source for translocations. Accordingly, our objectives were to:

1) assess the degree and pattern of geographic separation and genetic divergence among populations of the Northern Leopard Frog in Arizona;

2) identify isolated, relict populations that may require direct management intervention for persistence;

3) test for the extent of distribution of introduced eastern genotypes in

Arizona populations; and

4) discuss management strategies and conservation priorities for populations

in the region.

Materials and methods

Visual surveys for leopard frogs were conducted at all localities known or suspected to harbor Northern Leopard Frog populations in Arizona (sample collection sites presented in Fig. 4-1). Tissue samples were collected by clipping the tip of the third hind toe directly into a microvial containing 95% ethanol. To minimize the risk of infection, we applied an antibiotic/anesthetic ointment to the cut toes of sampled frogs before releasing them at their point of capture (Green 2001). Surgical instruments were sterilized between each frog to eliminate the risk of sample contamination and to reduce the risk of spreading diseases among frogs. These protocols were approved by the Northern Arizona University IACUC (permit # 07-003) and the Utah State University IACUC (permit # 1138). We targeted collection of a minimum of 20 samples per population. DNA was extracted from toe clips using either a standard chloroform extraction (Müllenbach et al. 1989) or a salting-out extraction method (Sunnucks and Hales 1996). The purified DNA was resuspended in a Tris-EDTA buffer (0.1M Tris, 0.1M EDTA, pH 9.0) and stored until use at $-80°$ C.

Sequence data for 786 base pairs of the mitochondrial ND1 gene (Hoffman and Blouin 2004a) were obtained for 1 to 15 individuals from each of the populations sampled, using the primers RpND1F and RpND1R (Wilson et al. 2008). These

sequences allowed the detection of eastern haplotypes, one of which had been

previously detected in one Arizona population (Hoffman and Blouin 2004a). Each 25

mL reaction contained 0.4 mmol/L of each primer, 160 mmol/L dNTPs, 1× polymerase

chain reaction (PCR) buffer, 2.5 mmol/L $MgCl_2$, 1 unit of Taq polymerase, and ~50 ng of

genomic DNA. PCR conditions consisted of 5 minutes of initial denaturation at 95° C;

followed by 35 cycles of 94° C for 60 seconds, 54° C for 60 seconds, and 72° C for 90

seconds; followed by a 5 minute final extension at 72° C. The PCR product was

visualized on a 0.7% agarose gel to check for product quantity and size. PCR products

were purified with QIAquick PCR purification kit (Qiagen) and sequenced with BigDye

chemistry (Applied Biosystems) on an ABI 3730 sequencer. Sequences were edited and

aligned with SeqMan II software. Sequences were generated in the forward direction first

and in the reverse direction if the sequence could not be confidently read throughout the

entire 786 base pair amplicon in the forward direction alone. TCS version 1.21 was used

to search for unique mitochondrial haplotypes and to examine the relationships among

those haplotypes using statistical parsimony (Clement et al. 2000).

To further screen the sampled frogs for eastern haplotypes, we developed an

economical restriction assay that could distinguish eastern and western haplotypes

without sequencing each individual. An ~800 base pair region of the ND1 gene was

amplified as described above for the sequencing. This amplified fragment was then

digested for 6 hours at 37° C using the restriction enzyme StyI. This enzyme digests

eastern fragments but not western fragments at a site that has been shown to be diagnostic

for the eastern and western clades in over 500 hundred samples collected from

throughout the range of the species (Chapter 3; Hoffman and Blouin 2004a). Digestion

was arrested by incubating at 70° C for 20 minutes and digested fragments were then

visualized on a 1.0% agarose gel.

Microsatellite markers developed by Hoffman et al. (2003) (Rpi100, Rpi101,

Rpi102, Rpi103, Rpi104, Rpi107, and Rpi108) and Hoffman and Blouin (2004b)

(RP193) were used to amplify eight microsatellite loci. PCR conditions were 2 minutes

of initial denaturation at 95° C, followed by 30 cycles of the following steps: 95° C for 30

seconds, annealing temperature for 30 seconds, and 72° C for 1 minute; followed by a 10

minute final extension at 72° C. Locus-specific annealing temperatures were as follows:

Rpi100: 52° C, Rpi101: 62° C, Rpi102: 50° C, Rpi103: 55°C, Rpi104: 56° C, Rpi107:

52° C, Rpi108: 52° C, and RP193: 56° C. The PCR product was visualized on a 0.7%

agarose gel to check for product quantity and size. PCR products were then analyzed on

an ABI 3100 or 3730 sequencer. Population-level analyses using microsatellite data were

conducted only on populations with 9 or more individuals sampled.

We used GENEPOP software (Raymond and Rousset 1995) to test whether

population genotypic proportions deviated from expectations under assumptions of

Hardy-Weinberg equilibrium (HWE), given observed allele frequencies. An exact test

over all loci was performed using the Markov Chain method with 1000 dememorization

steps, 100 batches per locus, and 1000 iterations per batch. We also used GENEPOP to

determine whether deviations could be attributed to heterozygote excesses or deficits.

Probabilities were interpreted using a Bonferroni correction for multiple population by

locus combinations for all such combinations that were polymorphic (n = 85).

Relationships among populations, based on allele frequency differences as

measured by Nei's (1972) genetic distance, were summarized by construction of a

UPGMA dendrogram using Tools for Population Genetic Analysis (TFPGA) software

and nodal strength assessed via bootstrapping over loci using 1000 replicates. Pairwise

population differentiation was assessed using an exact test of each population with a

sample size of at least 15 individuals using TFPGA software with 1000 dememorization

steps and ten batches of 2000 permutations per batch. Overall genetic structuring among

populations was assessed using Wier and Cockerham's θ_{ST} (Wier and Cockerham 1984;

Wier 1996), an estimator of F_{ST}, using TFPGA software, version 1.3 (Miller 1997).

Inbreeding within populations was assessed in TFPGA by calculating f, an estimate of

Wright's F_{IS} (Wier and Cockerham 1984; Wier 1996). The 95% confidence intervals for

the estimates of θ_{ST} and f were estimated by bootstrapping over loci with 1000 replicates.

Within-population genetic diversity was measured in three ways: average gene

diversity per locus, mean number of observed alleles per locus, and allelic richness (with

sample size rarified to n = 9 to allow comparison among populations with different

sample sizes). All diversity analyses were performed using FSTAT software (Goudet

1995).

We determined whether our populations bore the signature of a recent bottleneck

by employing the mode shift test of Luikart et al. (1998) and the sign test in

BOTTLENECK version 1.2.02 (Cornuet and Luikart 1997). In stable populations at

mutation-drift equilibrium, most alleles are expected to be at low frequencies. When a

population experiences a demographic bottleneck, low frequency alleles are preferentially

lost and the most common (modal) allele frequency categories are expected to shift such

that the lowest frequency category is no longer the most commonly observed category.

The mode shift test is a qualitative assessment of this distribution of allele frequencies.

The sign test is a quantitative analysis of the number of loci that have either an excess

or a deficiency of genetic diversity compared to the predicted level of diversity based on

the number of alleles detected. A population bottleneck typically results in an excess of

diversity for a given number of alleles. Only populations with sample sizes greater than

11 were used for this analysis.

In the Stoneman Lake area populations (Fig. 4-2), additional analyses were

performed to assess genetic structuring and to look for genetic signatures of introduced

eastern Northern Leopard Frogs. We used STRUCTURE software version 2.2 (Pritchard

et al. 2000) to assess these patterns. STRUCTURE uses a Bayesian approach to

determine the likelihood that each individual is a member of particular groups. We

defined two groups of hypothesized common ancestry using mitochondrial haplotype

(eastern vs. western, see results). Haplotypes were determined by sequencing or

restriction assay.   We thus assigned the number of potential subpopulations sharing

coancestry as two, one each for eastern and western mitochondrial haplotypes. Five

individuals whose mitochondrial haplotype was not known due to failure of the

mitochondrial sequence to amplify in PCR were designated as being of unknown

population origin. If assignment of nuclear microsatellites is consistent with

mitochondrial genes, we can infer that the frogs of eastern and western origins are not

interbreeding. Alternatively, if nuclear microsatellites within individuals is not consistent

with mitochondrial genes, we can infer that interbreeding is occurring between

individuals with eastern and western origins. STRUCTURE software was also used to

determine the most likely number of clusters (k) in the Stoneman Lake area populations,

without pre-assignment to mitochondrial groups or populations. If interbreeding between

mitochondrial groups is not extensive, one would expect the optimal value of k to be 2, representing nuclear affiliation with two major ancestral groups. An optimal k of 6 or more would infer that population geographic structure and gene flow patterns are influencing assignment to ancestral groups.

Results

Samples were collected from 258 frogs from 21 populations in this region (Table 4-1). Sample sizes ranged from 1 to 31 per population. Populations were distributed across northern Arizona, but were clustered in five general areas (Fig. 4-1). The Truxton Wash population was isolated from the others in western Arizona. The -9 Mile Draw population was located along the Colorado River near the northern border of Arizona. The Lyman Lake population was isolated near the eastern border of Arizona. Also isolated in eastern Arizona were the Hess Tank and Buckskin Tank populations, which were very near each other. The remaining 16 populations were spatially clustered in the Stoneman Lake area (Fig. 4-2).

Three mitochondrial haplotypes were found in this study. Two haplotypes were closely related and nested with Hoffman and Blouin's (2004a) western clade. One of these western haplotypes was found in all five population groups in our study. The second western haplotype was restricted only to the Stoneman Lake area populations, but was widespread among these populations south of and including 91-91C Tank. The third haplotype was included in Hoffman and Blouin's (2004a) eastern clade, a clade otherwise limited to populations in the eastern U.S. The eastern haplotype was limited to the

Stoneman Lake area populations, and was most common in the northern populations

(Fig. 4-3A). Restriction assays revealed similar patterns of mitochondrial haplotypes.

Most of the microsatellite loci we analyzed were highly polymorphic, ranging

from 5 to 11 alleles total across our samples (Table 4-2). Genetic diversity was high

across the populations in the Stoneman Lake area, intermediate at -9 Mile Draw and low

in the Truxton Wash population and in the Hess Tank/Buckskin Tank area by all three

measures of diversity (Table 4-3). Deviations from genotypic frequencies expected under

Hardy-Weinberg equilibrium were found in only two of the population-by-locus

combinations: Rarick Tank loci Rpi101 and Rpi107, using a Bonferroni correction for

multiple tests. The disequilibrium at Rpi101 was due to an excess of heterozygotes (P =

0.0022).

Clustering of populations based on microsatellite-based genetic distances

generally reflected broad spatial relationships (Fig. 4-4). Buckskin and Hess Tank were

very similar, but were collectively very distinct from the other populations. Truxton

Wash and -9 Mile Draw were likewise unique when compared to the other populations,

although they tended to cluster more closely with the Stoneman Lake populations than

the Buckskin and Hess Tank cluster, contrary to geographic expectations. The Stoneman

Lake populations formed a well supported cluster, as would be expected given their

hydrologic connectivity and geographic proximity.

Across all populations in this study, population-level structuring was very

pronounced (θ_{ST} = 0.315; 95% c.i. = 0.261 - 0.374). Consistent with the θ_{ST} results,

pairwise exact testing of all 11 populations where at least 15 individuals were sampled

indicated that each population was distinct from every other (P < 0.05) except for Buckskin and Hess Tanks (P = 0.32).

Seven populations (-9 Mile Draw, Buckskin Tank, Hess Tank, Rarick Tank, Roundup Park Tank, T-Bar Tank #2, and Truxton Wash) showed a modal shift towards higher allele frequency categories, suggesting past population bottlenecks (potentially including recent founder events) (Fig. 4-5). Sign tests performed using BOTTLENECK version 1.2.02 indicated that -9 Mile Draw, Butch Tank, Little Mormon Lake, North of Pratt Park, Rarick Tank, Roundup Park Tank, and T-Bar Tank #2 showed significant heterozygote excess under the infinite alleles mutation model, but only T-Bar Tank #2 showed excesses under the stepwise alleles model, both indications of significant recent bottlenecks. Under the two-phase model of mutation, generally considered the most accurate for use with microsatellites (Di Rienzo et al. 1994), Little Mormon Lake, Rarick Tank, and T-Bar Tank #2 showed indications of significant recent bottlenecks in the form of heterozygote excesses.

Among the Stoneman Lake populations, assignment of individuals to two groups on the basis of microsatellite allele frequencies did not correspond well with eastern vs. western mitochondrial haplotypes (Figs. 4-3 and 4-6). The distinct origins and current separation of eastern and western frogs were indicated by a microsatellite-based θ_{ST} of 0.273 between frogs with eastern and western mitochondrial haplotypes. However, program STRUCTURE assigned many individual frogs to two groups inconsistent with their mitochondrial haplotypes (Fig. 4-6), suggesting that gene flow has occurred between these two groups and mitochondrial haplotypes are not a reliable indicator of the majority of the genetic heritage of any given frog in this region. STRUCTURE analysis

for k optimality indicated that k > 6, further indicating that mitochondrial grouping was not the only driver of genetic affiliations among individuals. STRUCTURE analysis when k = 2 (without mitochondrial group pre-assignment) indicated a gradient of group membership (Fig. 4-3B) generally concordant with the gradient of mitochondrial types (eastern vs. western) (Fig. 4-3A).

Hoffman and Blouin (2004a) originally identified one eastern individual among a sample of ten frogs collected from the Roundup Park Tank area in 2001 (these samples were collected by S. MacVean, Arizona Game and Fish Department, pers. comm.). We intensively sampled Roundup Park Tank in 2007 and 2008, and collected tissue from 31 adult and subadult frogs. Of these 31 frogs, 17 had the eastern haplotype. This is a statistically significant increase in the proportion of frogs with eastern mitochondrial haplotypes in this area, the only site for which we have data from two samples separated in time (Fisher's Exact Test, P = 0.025).

Discussion

Broad patterns of genetic divergence among populations

Throughout the study area, genetic divergence among populations is generally high. This is not surprising given the great distances that separate most of the remaining populations. Several populations, especially Truxton Wash, Hess Tank, Buckskin Tank, and -9 Mile Draw, showed both reduced genetic diversity and loss of rare alleles, indicating persistently small population sizes and recent population bottlenecks. Hoffman and Blouin (2004b) measured genetic diversity in peripheral and central populations of the Northern Leopard Frog. Although the loci we used did not match

precisely with the loci they used, there is sufficient overlap that we can compare levels

of genetic diversity at a coarse level. Some of the populations in the Stoneman Lake area

had levels of diversity that were approximately equal to those of Hoffman and Blouin's

"interior" populations. Populations outside of the Stoneman Lake area all had genetic

diversity that was roughly equivalent to or below Hoffman and Blouin's "peripheral"

populations.

Most of the populations with sample sizes greater than 11 showed molecular

signatures of recent demographic bottlenecks, despite the low statistical power associated

with our relatively small sample sizes. Such signatures were somewhat inconsistent

across methodological approaches, but were generally congruent with low allelic richness

(Table 4-3). This pattern is perhaps not unexpected given the dramatic impacts on

riparian areas and springs during the last century, but it suggests a need for urgent

management attention to avoid the potentially negative synergistic effects of small

population sizes, population isolation, and inbreeding depression.

Buckskin Tank and Hess Tank were very similar to one another and very different

from the other populations in terms of microsatellite allele frequencies (Fig. 4-4). Both

may have become established by anthropogenic introduction from nearby populations in

the 1990s (Kimberling et al. 1996; Miller et al. 1999). Mitochondrial haplotypes from

these populations match those native to the region, so if these populations are the result of

a recent introduction then they were probably introduced from a source population in the

same region. Microsatellite allele frequencies have diverged from those of other native

populations in the region, perhaps due to founder effects or drift (exacerbated by small

population size and isolation). Inbreeding in these populations is so extreme that four

pairs of individuals and one triplet of individuals were identical at all eight

microsatellite loci, indicating that this population was either founded by very few

individuals from another source or had recently gone through an extreme population

bottleneck.

Stoneman Lake area and possible hybridization

In contrast to other population groups in Arizona, the Stoneman Lake area has

relatively low genetic divergence among populations and high genetic diversity within

populations. In part, this reflects the large effective population size in this area, which is

characterized by a complex of many ponds that are connected by some gene flow.

However, the elevated genetic diversity in the Stoneman Lake complex has likely been

augmented by introductions of eastern frogs. In samples collected between 1997 and

2001, Hoffman and Blouin (2004a) detected one individual frog that contained an eastern

mitochondrial haplotype, out of ten frogs sampled from Roundup Park Tank. They

suggested that this eastern individual might have been a liberated laboratory animal or

pet. Our data suggest that eastern haplotypes were present in the region at least as early

as 1994, and that they are currently well established. We reexamined samples collected

in 1994 by Kimberling et al. (1996) from Butch Tank: of 15 samples, two were the

eastern haplotype.

The eastern haplotype detected in the Stoneman Lake area is a haplotype found in

New York, New England, and adjacent areas of Canada in Quebec and Ontario. This is

an area that was historically (Gibbs et al. 1971) and is presently (Angela White,

Biologist/Product Developer, Carolina Biological Supply, pers. comm.) a source of

Northern Leopard Frogs for the commercial trade. Determining the exact timing of the introduction of these eastern genotypes is problematic with the data that are presently available. However, our data do show that eastern mitochondrial haplotypes are widespread in the Stoneman Lake area, and microsatellite data indicate that eastern and western genetic lineages are broadly introgressed in this area.

An alternative explanation for the presence of eastern mitochondrial haplotypes in the Stoneman Lake Area is that they are a relict haplotype from the Pliocene when eastern and western haplotype frogs interbred south of their current range. We feel this explanation is very unlikely for two reasons. First, F_{ST} values between frogs with eastern and western mitochondrial haplotypes indicate that although some introgression has occurred, microsatellite alleles are not randomly assorted among eastern and western mitochondrial haplotypes as would be expected if these haplotypes had been present and interbreeding since the Pliocene. Second, the eastern haplotype that is present in Arizona is from a remote group of populations that is not likely to have been interbreeding naturally with Arizona frogs.

This scenario of recent anthropogenic introduction is based on some assumptions and does not entirely eliminate the possibility that eastern genotypes in the Stoneman Lake area are relicts of a pre-Pleistocene contact between eastern and western lineages in this area. To more rigorously test this hypothesis, it would be necessary to sequence several nuclear genes in addition to the mitochondrial genes already sequenced. By comparing coalescence times among DNA sequences, estimates of the timing of introduction could be generated and hypotheses of the timing of introduction, i.e. pre-Pleistocene or Holocene, could be tested.

Assessing the degree of introgression on the basis of allele frequencies, such as in the assignment test displayed in Fig. 4-6, would be a powerful approach to evaluate introgression between eastern and western frogs in the Stoneman Lake area if allele frequencies for native frogs and those of introduced frogs were very different. However, we have no reference data on allele frequencies in putatively introduced "eastern" frogs, and this method may not have much power to detect introgression if eastern frogs had allele frequencies that were similar to the native western frogs.

Management Implications

It appears very likely that Northern Leopard Frogs in the Stoneman Lake area have significant eastern genetic influence from frogs introduced in the recent past. This potentially complicates the management of the species in Arizona, and raises some difficult questions. The remote and isolated populations such as Truxton Wash and Hess Tank/Buckskin Tank have low genetic diversity and might benefit from the addition of genetic diversity from other populations. All other factors being equal, the obvious choice for a source population of genetic diversity would be populations in the Stoneman Lake area. Populations in this area have the highest genetic diversity of any populations in Arizona, and because this is a complex with gene flow among populations, this area is most likely to tolerate the export of individuals for relocation or genetic exchange programs. However, the elevated genetic diversity of this area is likely due to interbreeding with non-native individuals, evidently introduced from the northeastern United States or southeastern Canada. Spreading individuals from this healthy population to other areas would mix non-native genotypes into pure native populations,

potentially leading to outbreeding depression and the swamping of locally adapted

genotypes. If such introductions were successful, they would be irreversible.

Maintenance of local adaptation is not and perhaps should not always be the only

concern in the management of endangered and threatened species. If remote localized

populations are threatened with extinction because of small population sizes and the lack

of genetic diversity, it may be more important to maintain some form of the species in

question than to try to preserve genetic integrity at the peril of the population (Allendorf

et al. 2001). This was the reasoning for hotly contested translocation of cougars (*Puma

concolor stanleyana*) from Texas to the range of the Florida Panther (*Puma concolor

coryi*), an action that was later judged to be critical to the recovery of the subspecies in

Florida (Pimm et al. 2006). However, mixing of native genomes with nonnative genomes

is a serious concern in the management of endangered species. Given the deep genetic

divergences between eastern and western Northern Leopard Frogs, it is likely that they

will one day again be recognized as distinct subspecies, or perhaps even distinct species,

as they once were in the past (Chapter 3). The translocation of individuals within a

species, but between ranges of subspecies, has caused significant conservation concerns

for species before (Metcalf et al. 2007), and it would be wise to avoid spreading

nonnative haplotypes unless absolutely necessary. Given the opportunity to avoid the

risk of outbreeding depression in these already tenuous populations, it is apparent that a

preferred alternative would be to translocate individuals from other populations in the

vicinity, such as those in Utah and Colorado (Chapter 3).

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Table 4-1: *Rana pipiens* sample sizes included in this study. All samples were also

genotyped using microsatellite loci (except 13 individuals from Stoneman Lake), and all

samples not sequenced were subject to a diagnostic restriction assay to identify

haplotypes from the eastern U.S. "Samples sequenced" refers to the number of samples

for which 786 bp of sequence data from the mitochondrial ND1 gene was obtained

(Hoffman and Blouin 2004a). "Percent eastern" refers to the percentage of samples that

matched the eastern U.S. mitochondrial haplotypes (Hoffman and Blouin 2004a), as

assessed either by sequencing or restriction assay.

Locality Name and Location	Total Samples	Samples Sequenced mtDNA	Percent eastern haplotype
Colorado River; northern AZ			
-9 Mile Draw	14	5	0
Southeast of Stoneman Lake			
Buckskin Tank	15	6	0
Hess Tank	15	5	0
Eastern AZ			
Lyman Lake	2	2	0
Northwestern AZ			
Truxton (Spring and Wash)	25	5	0
Stoneman Lake Area			
1) 91-91C Tank	1	1	100
2) Brady Tank	2	2	0

3) Brolliar Park Tank	2	2	100
4) Burn Tank	4	4	75
5) Butch Tank	16	6	13
6) Camp Tank	3	3	67
7) Little Mormon Lake	23	9	83
8) Mulholland Tank	1	1	0
9) New Tank	3	3	100
10) North of Pratt Park	19	5	11
11) Pratt Park	9	5	0
12) Rarick Tank	23	5	0
13) Roundup Park Tank	31	15	55
14) Stoneman Lake	24	5	4
15) T-Bar Tank #2	25	10	43
16) Tie Park Tank	2	2	0

Table 4-2: Total number of alleles found for each microsatellite locus across

populations.

_____

Microsatellite Locus	Total Alleles
Rpi100	8
Rpi101	8
Rpi102	8
Rpi103	11
Rpi104	5
Rpi107	11
Rpi108	6
RP193	9

_____

Table 4-3: Estimates of genetic diversity for each population based on microsatellite analyses. Number of samples genotyped at microsatellite loci (n), average gene diversity per locus (gene diversity), total number of observed alleles (# Alleles), and allelic richness, rarified to n = 9, are provided. Populations with sample sizes under 9 are not shown.

| Population	n	Gene Diversity	# Alleles	Allelic Richness
-9 Mile Draw	14	0.508	23	21.7
Buckskin Tank	15	0.219	15	14.5
Hess Tank	15	0.194	12	11.8
Truxton Wash	25	0.298	14	13.4
Stoneman Lake Populations				
Butch Tank	16	0.643	38	34.3
Little Mormon Lake	22	0.570	28	24.4
North of Pratt Park	19	0.619	33	29.1
Pratt Park	9	0.586	29	29.0
Rarick Tank	23	0.551	28	25.2
Roundup Park Tank	31	0.613	33	29.2
Stoneman Lake	11	0.603	37	35.0
T-Bar Tank #2	25	0.587	24	23.2
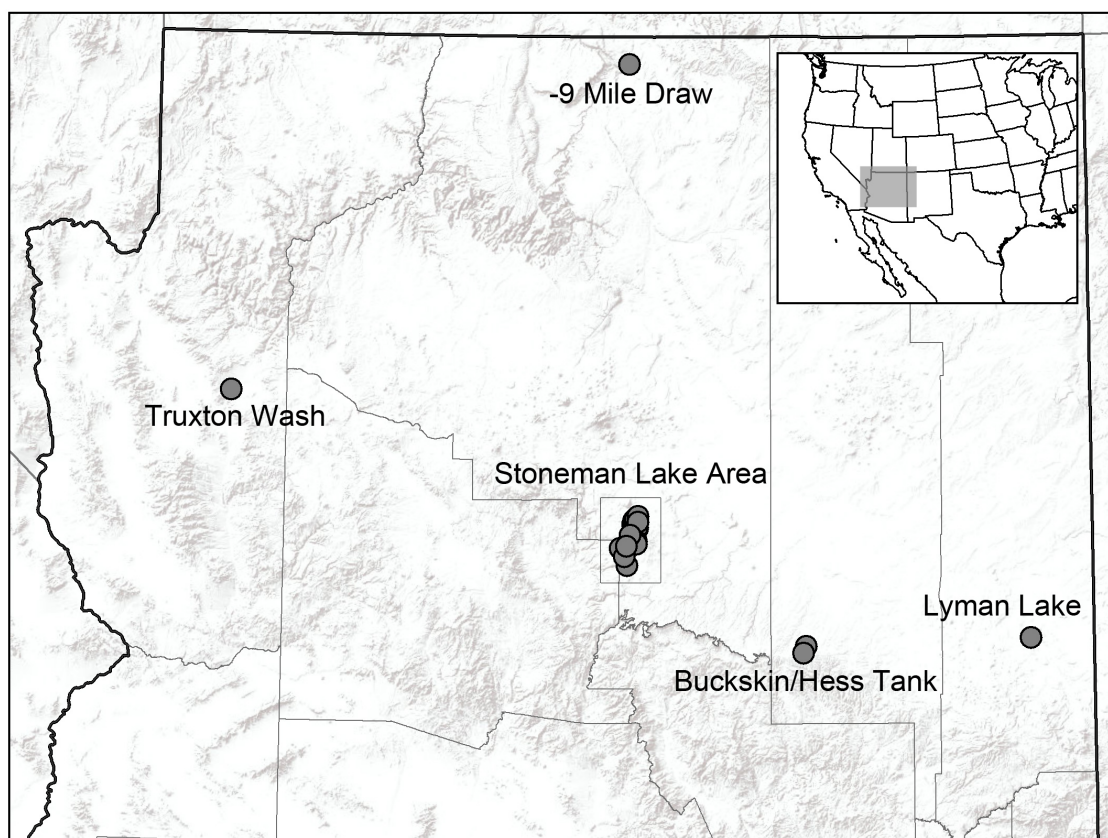
Fig. 4-1: Location of sampled populations included in this study. Distance from Truxton Wash to Lyman Lake is 405 km. The cluster of populations in the Stoneman Lake area at the center of the map is enlarged in Fig. 4-2.
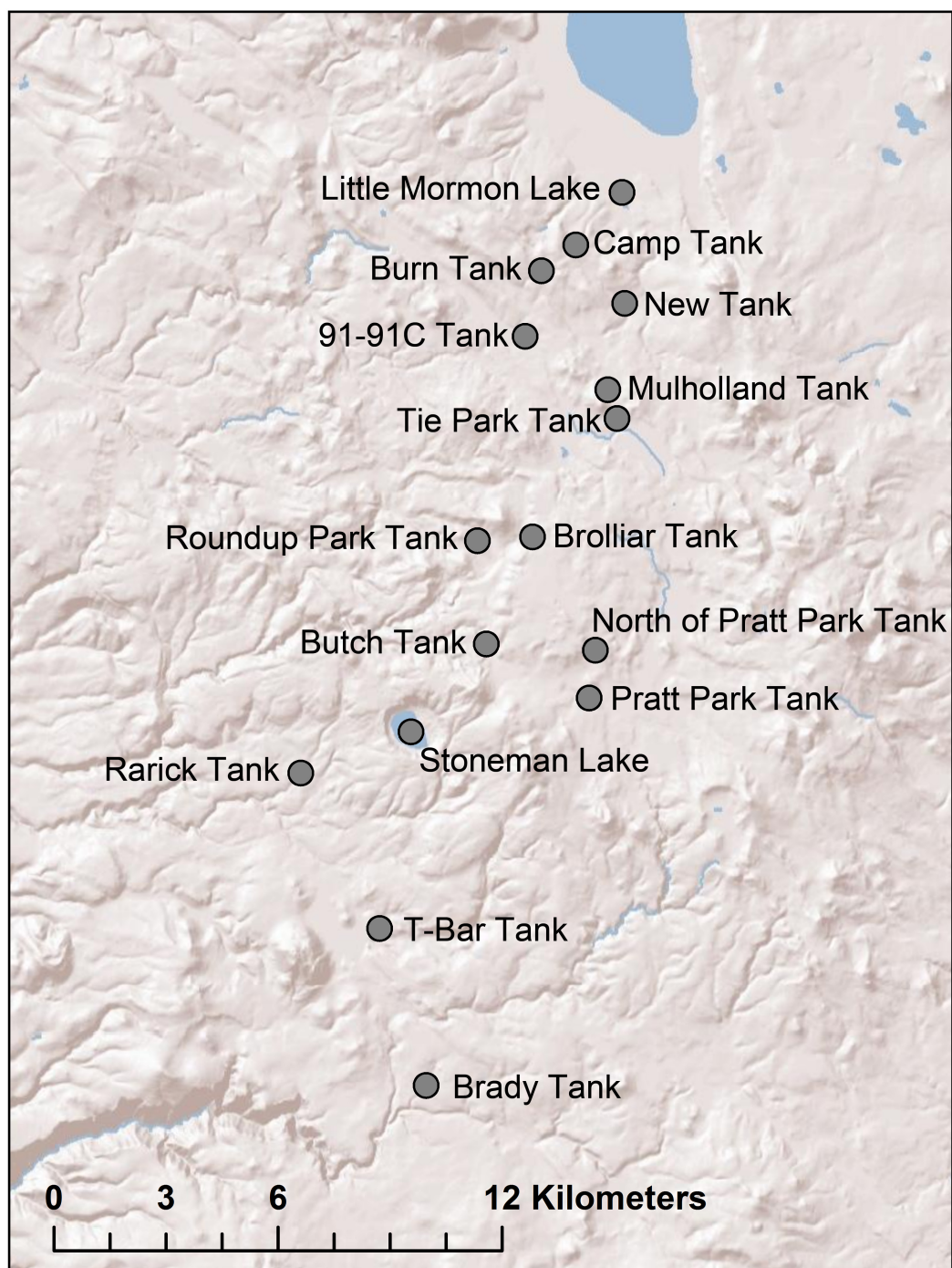
Fig. 4-2: Populations sampled in the Stoneman Lake area (the central cluster of populations in Fig. 4-1).
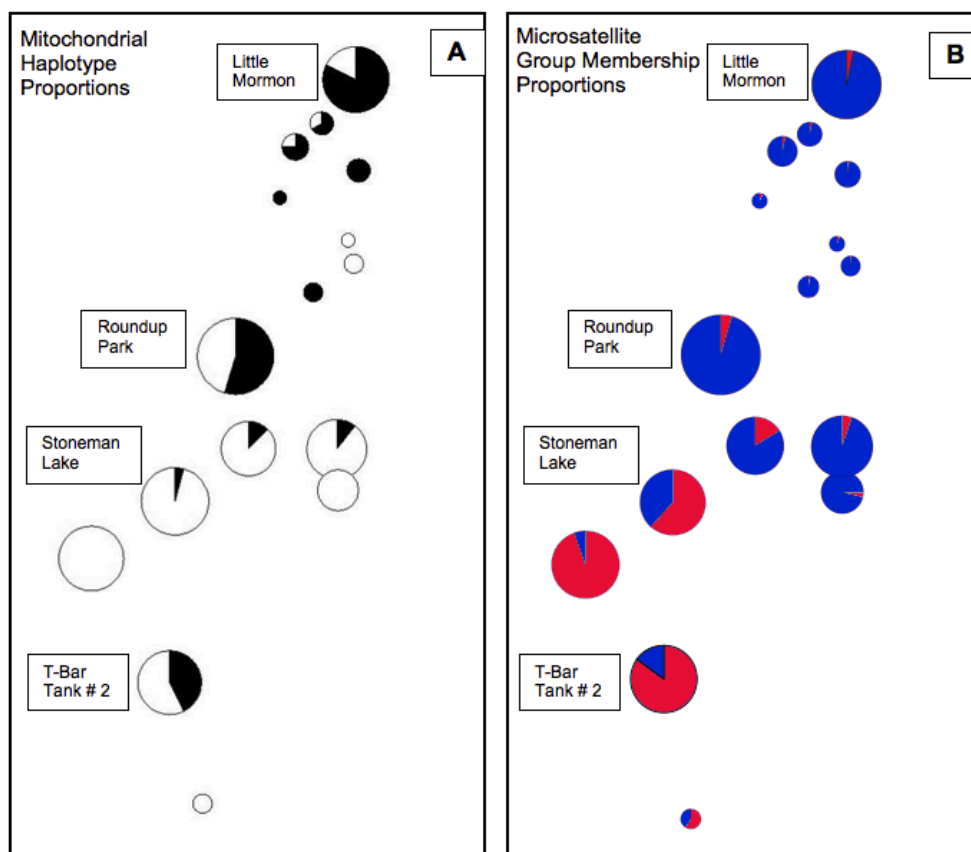
Fig. 4-3: A) Distribution of eastern and western mitochondrial haplotypes in the Stoneman Lake area. Black indicates relative proportion of individuals with the eastern haplotype, and white indicates proportion of western haplotypes. Results of sequencing and restriction assays are combined here. B) Distribution of microsatellite group membership based on STRUCTURE analysis using k = 2 without mitochondrial group pre-assignment. Blue represents proportion of STRUCTURE group 1 membership, and red represents STRUCTURE group 2 membership, averaged across individuals. In both A and B, circles represent sampled populations with size of circle proportional to sample sizes of populations (n = 1 to 31). See Fig. 4-2 for other site names.

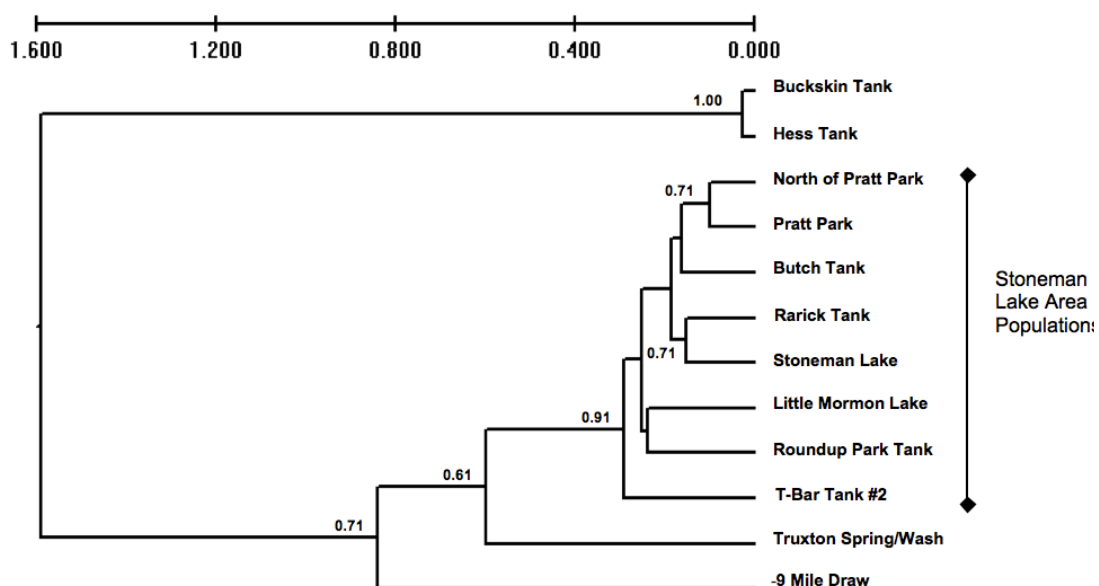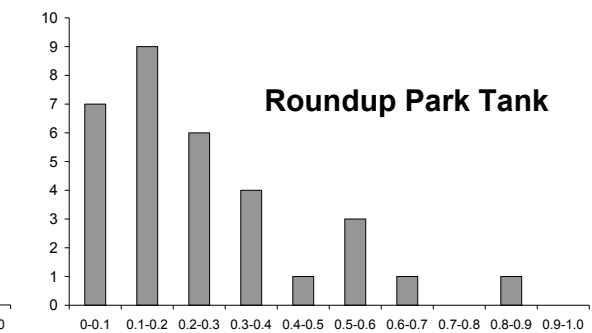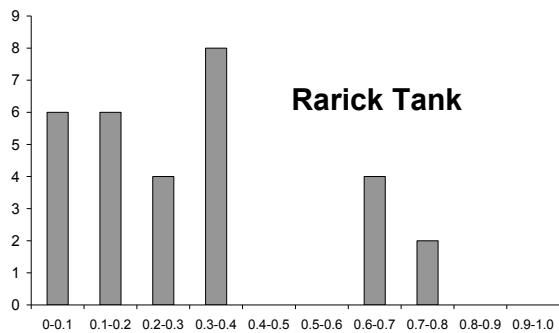Fig. 4-4: UPGMA dendrogram of Nei's (1972) distances based on microsatellite allele frequencies. Proportion of bootstrap replicates regenerating each node with greater than 30% frequency is provided. Bootstrap values less than 50% should be interpreted with caution.
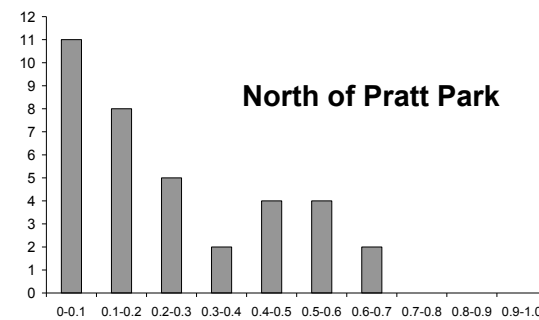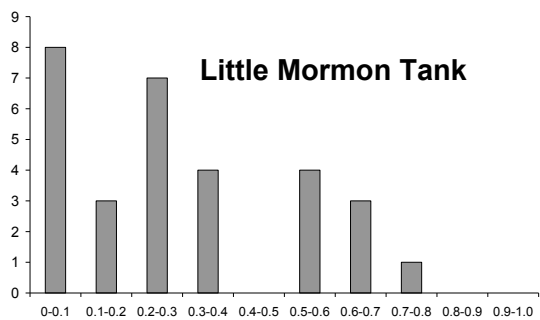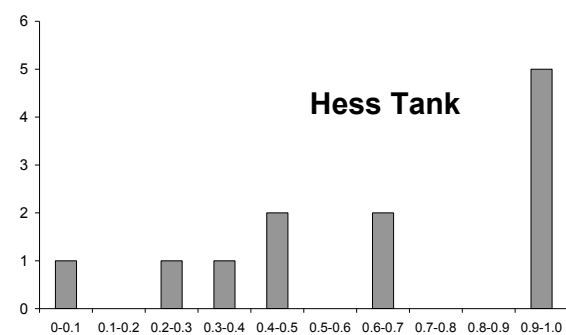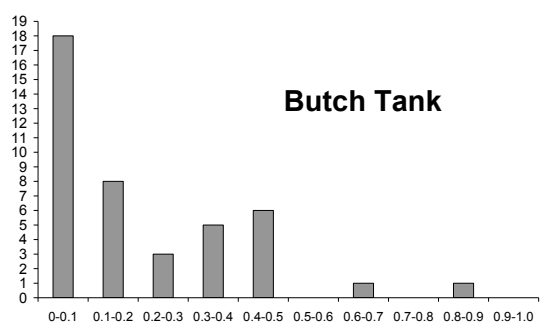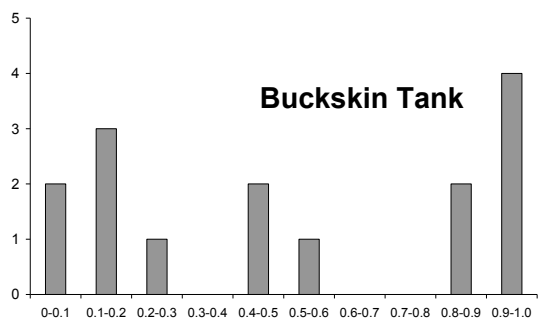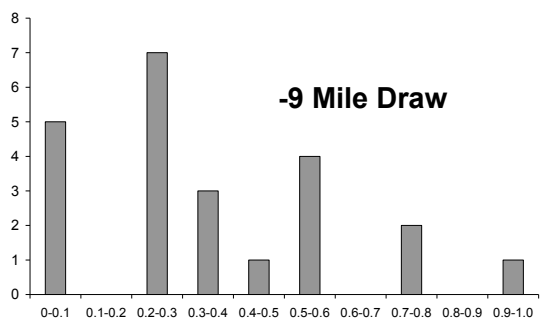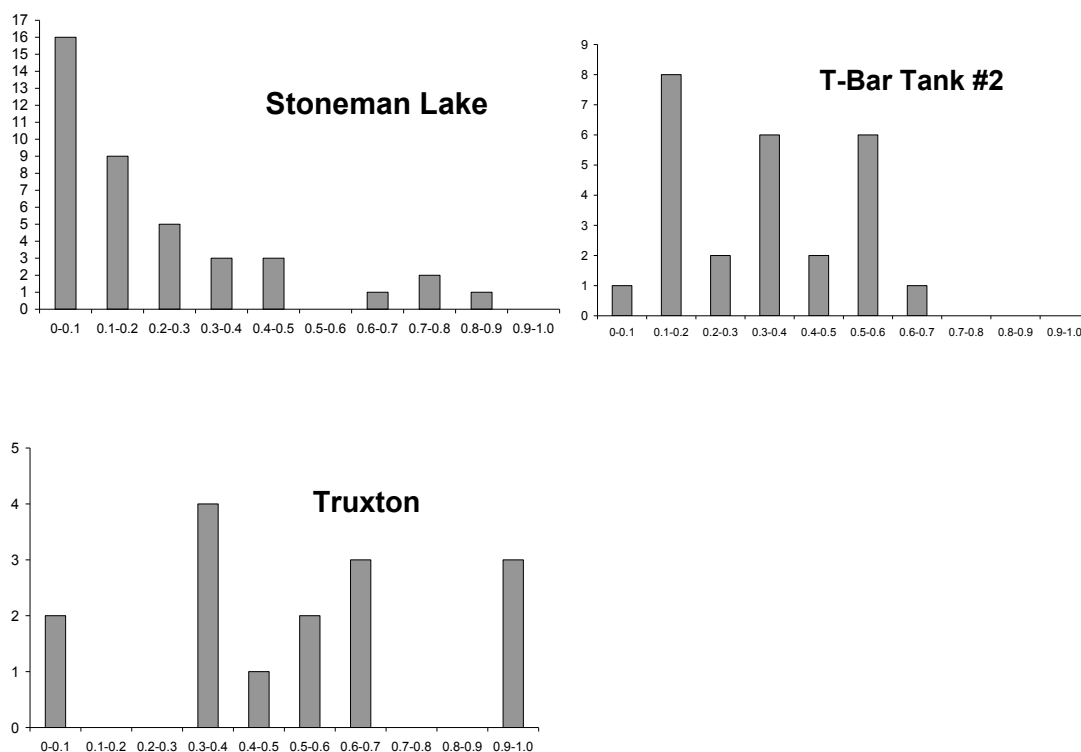
Fig. 4-5: Allele frequency histograms for 11 populations with at least 15 individuals sampled. Distributions skewed to the left (i.e. with a modal allele frequency of 0.01-0.1) indicate populations without a significant recent bottleneck. Distributions where the mode is not 0.01-0.1 indicate populations that have likely gone through recent bottlenecks.

Western mtDNA **Eastern mtDNA**
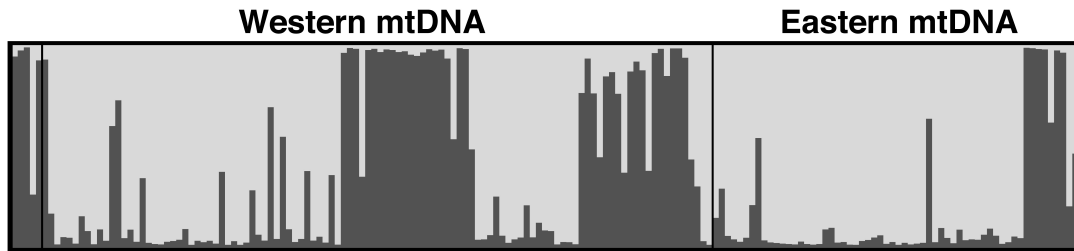
Fig. 4-6: Probability of individual membership in one of two clusters detected by STRUCTURE analysis (k = 2), based on microsatellite allele frequencies. Individuals are represented as vertical bars. The two clusters defined by STRUCTURE analysis are presented as light or dark gray. An individual with a 90% dark gray bar, for example, has a 90% probability of being derived from the 'dark gray' STRUCTURE cluster and a 10% probability of being derived from the 'light gray' STRUCTURE cluster. Individuals are divided into those with western mitochondrial haplotypes (left) and those with eastern mitochondrial haplotypes (right). Five frogs whose mitochondrial DNA failed to amplify in the PCR are grouped together at the far left. If individuals with a particular haplotype (eastern or western) also represented a distinct group with respect to nuclear alleles, we would expect very high assignment fidelity within mitochondrial groups (i.e. individuals with western mitochondrial haplotypes would appear as mostly light gray lines and individuals with eastern mitochondrial haplotypes would appear as mostly dark gray lines.)

CHAPTER 5

CONCLUSIONS

Current predictions of genetic diversity across species' ranges are coarse, usually simply assuming lower diversity in edge populations and higher diversity in central populations as a consequence of the abundant-center distribution and the known consequences of population size and connectivity on genetic diversity (Gaston 2003; Eckert et al. 2008). In natural populations, however, the distribution of genetic diversity is rarely so simple. At the mechanistic level, population genetic diversity is driven by the four forces of mutation, migration, selection, and drift. However, integrating these four factors into an understanding of the demographic history of a population is a complicated task, and thus our ability to predict genetic diversity across species' ranges is, and will probably remain, imperfect.

A first step in improving our ability to predict genetic diversity in populations is to compare alternative hypotheses in explaining current levels of genetic diversity (Eckert et al. 2008). In Chapter 2, we tested several alternative hypotheses to explain genetic diversity across the range of the Northern Leopard Frog, and to explain why some edge populations may not show reduced genetic diversity. We also examined the pattern of genetic diversity continuously from range center to edge in two transects that differed in the nature of their edges. We found that genetic diversity was reduced in edge populations relative to central populations, but was not reduced in populations in glaciated areas versus unglaciated ones. Position at range edge had a stronger effect in reducing diversity than recent colonization in this species. Further, we found that genetic

diversity declined towards the range edge in a transect into increasingly fragmented habitat, but not in the edge characterized by more continuous habitat. Taken together, these results provide evidence that genetic diversity in this species is reduced by position at the range edge, but that this effect can vary among individual transects, depending on the nature of the habitat at a particular edge.

In Chapter 3, we tested whether the phylogeography of this species that was known from mitochondrial DNA was congruent with the phylogeography shown by nuclear DNA. A genetic boundary at the Mississippi River, USA, had been suggested for the Northern Leopard Frog on the basis of limited geographic sampling of a mitochondrial gene (Hoffman and Blouin 2004a). However, mitochondrial DNA represents a very small part of the genome and is not necessarily indicative of patterns in nuclear DNA. We tested the hypothesis that eastern and western populations were separated by a distinct genetic boundary by sequencing mitochondrial DNA more extensively across the range, including focused sampling in the zone of hypothetical introgression, and by analyzing four nuclear sequences and seven microsatellite loci. We confirmed previous results that eastern and western populations have unique mitochondrial sequences that are deeply divergent and which overlap only in a narrow region around the Mississippi River. Nuclear sequences also showed divergent eastern and western lineages in some cases but with a broader zone of geographic overlap. Microsatellite data corresponded closely to mitochondrial data, differing between east and west and changing abruptly near the Mississippi River. These data collectively demonstrate that eastern and western clades of this species introgress in some markers but are distinct and defined by clear and narrow boundaries in others. We demonstrate

that the Mississippi River forms an important, albeit somewhat permeable, boundary between genetic lineages in this species in the United States. These results help clarify some of our findings in Chapter 2, where we found a peak of genetic diversity in the vicinity of the Mississippi River. In this species, genetic diversity in that region is elevated by the convergence of two populations that were isolated from one another in the Pleistocene.

In Chapter 4, we narrowed the geographic focus to northern Arizona, where a previous study had indicated the presence of at least one individual from the eastern part of the range. We analyzed genetic diversity within and genetic divergence among populations of Northern Leopard Frog throughout Arizona to assess the current levels of isolation among these populations. We also analyzed mitochondrial DNA to place these populations into a larger phylogenetic framework and to determine whether any populations contained genetic material not native to the region. We found a high level of genetic divergence between populations, and low genetic diversity in the isolated populations. Populations in the Stoneman Lake area had unusually high genetic diversity and relatively high gene flow among populations. However, this complex of populations also contained a mitochondrial haplotype that was from eastern frogs, probably representing the introduction of released pets or laboratory animals. These eastern haplotypes were well integrated into this complex of ponds, and probably contributed to the high genetic diversity in this area. Genetic diversity in the outlying populations was low and showed signs of recent bottlenecks, and could probably benefit from additional genetic diversity. On the surface, Stoneman Lake would appear to be an ideal source of frogs for translocations into the genetically depauperate populations elsewhere in

Arizona. However, in light of the large differences between the genetics of the eastern and western clades within this species (Chapter 3), and the unpredictable consequences outbreeding, supplementing genetic diversity in these native populations with artificial gene flow from the Stoneman Lake area would only be recommended if no alternatives exist.

In conclusion, each of the studies presented here were conducted with the goal of illuminating the process driving genetic diversity in natural populations. It is hoped that this work will not only inform the management of Northern Leopard Frogs, but also to provide some insight into the mechanisms that drive genetic diversity generally in natural populations.

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CURRICULUM VITAE

RYAN P. O'DONNELL

(April 2012)

EDUCATION

Ph.D. Candidate, Ecology. 2006-present. Dissertation title: "Genetic diversity and genetic structuring at multiple spatial scales across the range of the northern leopard frog, *Rana pipiens*," with advisor Karen E. Mock. Utah State University. 4.00 GPA.

M.S., Zoology. 2004. Thesis title: "Behavioral adaptations and the minimization of reproductive costs in the male red-sided garter snake, *Thamnophis sirtalis parietalis*," with advisor Robert T. Mason. Oregon State University. 3.95 GPA.

B.S., Zoology. 1999. Honors thesis title: "Effects of moonlight and other environmental variables on the calling behavior of barred owls and great horned owls," with advisor Michelle P. Scott. University of New Hampshire. 3.77 GPA, University Honors in Zoology, Presidential Scholar, Summa Cum Laude.

PROFESSIONAL EXPERIENCE

Biological Technician. Frontier Corporation USA. 2011. Conducted point counts for birds and visual surveys for breeding raptors and lekking grouse.

Biologist. United States Geological Survey, Southwest Biological Science Center. 2010-current. Conducted population genetic and phylogeographic analyses of Northern Leopard Frog populations. Prepared internal reports and peer-reviewed publications.

Ecological Research Analyst. Washington Department of Fish and Wildlife, Habitat Program, Science Division. 2004 - 2006. Analyzed data regarding amphibian habitat use and sampling techniques. Published findings in peer-reviewed journals. Managed Microsoft Access database. Coordinated a study of the effects of shade on primary productivity and amphibian growth in streams in managed forests. Collaborated with private landowners and state and federal agencies. Supervised a field crew of 2-4 scientific technicians.

Scientific Technician II. Washington Department of Fish and Wildlife, Habitat Program, Science Division. 2004. Studied amphibian associations with headwater seeps. Constructed and checked drift fences and funnel traps, surveyed for stream and terrestrial amphibians. Contributed to experimental design. Analyzed data and published results in peer-reviewed journals.

PEER-REVIEWED PUBLICATIONS

O'Donnell, R.P. 2004. Effects of environmental conditions on owl responses to broadcast calls. Transactions of the Western Section of the Wildlife Society 40(1):101-106.

O'Donnell, R.P., N.B. Ford, R. Shine, & R.T. Mason. 2004. Male red-sided garter snakes, *Thamnophis sirtalis parietalis*, determine female mating status from pheromone trails. Animal Behaviour 68(4):677-683.

O'Donnell, R.P., R. Shine, & R.T. Mason. 2004. Seasonal anorexia in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. Behavioral Ecology and Sociobiology 56(5):413-419.

Shine, R., **R.P. O'Donnell**, T. Langkilde, M.D. Wall, & R.T. Mason. 2005. Snakes in search of sex: the relation between mate-locating ability and mating success in male garter snakes. Animal Behaviour 69(6):1251-1258.

O'Donnell, R.P. & S.J. Arnold. 2005. Evidence for selection on thermoregulation: effects of temperature on embryo mortality in the garter snake *Thamnophis elegans*. Copeia 2005(4):930-934.

O'Donnell, R.P., E.P. Urling, C. Sato, & M.P. Hayes. 2005. First record of a masked shrew (*Sorex cinereus*) in the Willapa Hills, Washington. Northwestern Naturalist 86(3):154-156.

Hoyer, R.F., **R.P. O'Donnell**, & R.T. Mason. 2006. Current distribution and status of sharp-tailed snakes (*Contia tenuis*) in Oregon. Northwestern Naturalist 87(3):195-202.

O'Donnell, R.P. & R.T. Mason. 2007. Mating is correlated with a reduced risk of predation in female red-sided garter snakes, *Thamnophis sirtalis parietalis*. American Midland Naturalist 157(1):235-238.

O'Donnell, R.P. 2007. Erroneous historical records of climbing salamanders (*Aneides*) in Washington State, U.S.A. Northwest Science 81(3):239-245.

O'Donnell, R.P., K. Staniland, & R.T. Mason. 2007. Experimental evidence that oral secretions of northwestern ring-necked snakes (*Diadophis punctatus occidentalis*) are toxic to their prey. Toxicon 50(6):810-815.

O'Donnell, R.P., T. Quinn, M.P. Hayes, & K.E. Ryding. 2007. Comparison of three methods for surveying amphibians in forested seep habitats in Washington State. Northwest Science 81(4):274-283.

O'Donnell, R.P. 2008. Terrestrial foot-paddling by a glaucous-winged gull. Western Birds 39(1):33-35.

O'Donnell, R.P. & D. McCutchen. 2008. A sharp-tailed snake (*Contia tenuis*) in the San Juan Islands: Western Washington's first record in 58 years. Northwestern Naturalist 89(2):107-109.

Latta IV, L.C., **R.P. O'Donnell**, & M.E. Pfrender. 2009. Vertical distribution of *Chlamydomonas* changes in response to grazer and predator kairomones. Oikos 118(6):853-858.

O'Donnell, R.P., S.R. Supp, & S.M. Cobbold. 2010. Hindrance of conservation biology by delays in the submission of manuscripts. Conservation Biology 24(2):615-620.

O'Donnell, R.P. & A.P. Rayburn. 2011. Biases in the protection of peripheral anuran populations in the United States. Herpetological Conservation and Biology 6(1):91-98.

O'Donnell, R.P. and C.H. Richart. 2012. Diet of the Columbia Torrent Salamander, *Rhyacotriton kezeri* (Caudata: Rhyacotritonidae): Linkages between aquatic and terrestrial ecosystems in forested headwaters. Northwestern Naturalist 93(1):17-22.

O'Donnell, R.P. and K.E. Mock. *In press*. Two frog species or one? A multi-marker approach to assessing the distinctiveness of genetic lineages in the Northern Leopard Frog, *Rana pipiens*. Conservation Genetics.

O'Donnell, R.P. *In review.* Birder envy: Harnessing the power of a global network of citizen herpetologists. Herpetological Review.

REPORTS

MacCracken, J.G, **R.P. O'Donnell**, & M.P. Hayes. 2006. Effects of vegetation retention on stream-associated amphibians: some pre-treatment data. Report to the Cooperative Monitoring, Evaluation, and Research Committee of the Washington Department of Natural Resources, Olympia, Washington. 55 pp.

O'Donnell, R.P., C.M. Culumber, & K. Mock. 2007. Population fragmentation and conservation genetics of Northern Leopard Frogs (*Rana pipiens*) in the Glen Canyon / Canyonlands Region: 2007 progress report. Report to the Southwest Biological Science Center, United States Geological Survey, Flagstaff, Arizona. 4 pp.

Drost, C., L. Gelczis, **R.P. O'Donnell**, K. Mock, J. Baker, & C.M. Culumber. 2008. Population status and viability of leopard frogs (*Rana pipiens*) in Grand Canyon, Glen Canyon, and Canyonlands. Report to the Bureau of Reclamation, Glen Canyon National Recreation Area, and Grand Canyon National Park. 42 pp.

O'Donnell, R.P. & K. Mock. 2008. Patterns of Genetic Diversity and Divergence in Northern Leopard Frogs (*Rana pipiens*) in the Glen Canyon and Canyonlands Regions of Southern Utah and Northern Arizona. Report to the Southwest Biological Science Center, United States Geological Survey, Flagstaff, Arizona. 20 pp.

O'Donnell, R.P. & K.E. Mock. 2009. Patterns of Genetic Diversity and Divergence in Northern Leopard Frogs (*Rana pipiens*) in Northern Arizona. Report to the Southwest Biological Science Center, United States Geological Survey, Flagstaff, Arizona. 20 pp.

SELECTED GRANTS, ASSISTANTSHIPS, & AWARDS

Graduate Research Assistant of the Year. 2012. Utah State University College of Natural Resources.

Terri Lynn Steel Award. 2011. Utah State University College of Natural Resources.

U.S. Geological Survey. 2010. Genetic assessment of spatial and biological boundaries in the Northern Leopard Frog (currently proposed as a federally threatened species). Charles Drost, Karen Mock, and Ryan O'Donnell. $29,596.

Utah Division of Wildlife Resources, Endangered Species Mitigation Fund. 2010. Genetic assessment of Northern Leopard Frogs in Utah: Diversity, divergence, and hybridization. Karen Mock, Ryan O'Donnell, and Charles Drost. $24,024.

Bureau of Reclamation, Activities to Avoid Jeopardy grant program. 2010. Nuclear phylogenetics and the extent of introgression between western and eastern clades of the Northern Leopard Frog, *Lithobates* (= *Rana*) *pipiens*. Ryan P. O'Donnell, Karen Mock, and Charles A. Drost. $41,806.

Ecology Center Assistantship. Utah State University Ecology Center. 2010. $12,000.

Ecology Center Research Support Award, Utah State University Ecology Center. 2010. Testing for evidence of reproductive isolation between two clades of the Northern Leopard Frog, *Rana pipiens*. $4,000.

Graduate Student Senate Research and Projects Grant, Utah State University Graduate Student Senate. 2009. Why do peripheral populations have reduced genetic diversity? Testing a hypothesis of range expansion and contraction. $800.

Ecology Center Research Support Award, Utah State University Ecology Center. 2009. Why do peripheral populations have reduced genetic diversity? Part 2: Testing predictions of range edge types. $3,000.

Ecology Center Research Support Award, Utah State University Ecology Center. 2008. Why do peripheral populations have reduced genetic diversity? $4,000.

Willard L. Eccles Graduate Fellowship, Utah State University. 2006-2009. Circa $106,000.

Graduate Assistantship in Areas of National Need, U.S. Department of Education. 2000-2003. Complex Behaviors. Circa $100,000.

Gaige Award, American Society of Ichthyologists and Herpetologists. 2001. The effects of temperature on sex ratio in garter snakes (Genus: *Thamnophis*). $500.

Undergraduate Research Award, Undergraduate Research Opportunities Program, University of New Hampshire. 1999. The effects of moonlight and other environmental factors on the calling behavior of barred owls (*Strix varia*) and great horned owls (*Bubo virginianus*). $400.

SELECTED PROFESSIONAL PRESENTATIONS

O'Donnell, R.P., C.A. Drost, & K.E. Mock. 2011. Two frogs or one? Examining the evolution of Northern Leopard Frogs using multiple genetic markers. The Wildlife Society, Waikoloa, HI.

Drost, C.A., **R.P. O'Donnell**, & K.E. Mock. 2011. Population status and population genetics of Northern Leopard Frogs (*Rana pipien*s) on the southern Colorado Plateau. Biennial Conference of Research on the Colorado Plateau. Flagstaff, AZ.

O'Donnell, R.P., K.E. Mock, & C.A. Drost. 2011. Population status and population genetics of Northern Leopard Frogs in Arizona. Current Research on Herpetofauna of the Sonoran Desert V. Tucson, AZ.

Supp, S.R., **R.P. O'Donnell**, & S.M. Cobbold. 2010. Hindrance of conservation biology by delays in the submission of manuscripts. Eleventh annual Student Conference on Conservation Science. Cambridge, England. (Poster)

O'Donnell, R.P., S.R. Supp, & S.M. Cobbold. 2010. Conservation biology is hindered by delays in the submission of manuscripts. Society for Northwestern Vertebrate Biology. Medford, OR. (Plenary talk, Best Student Presentation award)

Drost, C.A., **R.P. O'Donnell**, S. MacVean, & K. Mock. 2010. Population status and genetics of Northern Leopard Frogs in northern Arizona. Southwest Section of The Wildlife Society.

O'Donnell, R.P. & A.P. Rayburn. 2009. Frogs on the edge: Predicted and actual biases in the protection of peripheral populations in the United States. Society for Northwestern Vertebrate Biology. Stevenson, WA.

Richart, C.H., M.P. Hayes, **R.P. O'Donnell**, K. Young, R. Crawford, & C. Maynard. 2007. Diet of terrestrial forest-dwelling amphibians in Washington. Society for Northwestern Vertebrate Biology. Victoria, British Columbia, Canada.

O'Donnell, R.P. 2004. Do individual differences in response to environmental conditions affect detection rates of barred and great horned owls in call-response surveys? Western Section of the Wildlife Society. Rohnert Park, CA.

O'Donnell, R.P., R. Shine, & R.T. Mason. 2003. Seasonal shift in breeding and feeding in the male red-sided garter snake, *Thamnophis sirtalis parietalis*. Chemical Signals in Vertebrates X. Corvallis, OR. (Poster)

O'Donnell, R.P., N.B. Ford, R. Shine, & R.T. Mason. 2003. Trailing pheromones in the red-sided garter snake (*Thamnophis sirtalis parietalis*) reveal reproductive status. Animal Behavior Society. Boise, ID.

Westphal, M.F. & **R.P. O'Donnell**. 2002. Behavioral modification of color pattern in garter snakes. American Society of Naturalists. Banff, Alberta, Canada.

INVITED SEMINAR PRESENTATIONS

Wildlife Science Seminar Series, College of Forest Resources, University of Washington, Seattle, WA. 2004.

Black Hills State University, Spearfish, SD. 2008.

Southern Utah University, Cedar City, UT. 2010.

COURSES TAUGHT

Instructor. 2012. Ornithology, BIOL 5560. Utah State University.

Teaching Assistant. 2010. Evolutionary Biology, BIOL 5250. Utah State University.

Teaching Assistant. 2009. Evolutionary Biology, BIOL 5250. Utah State University.

Teaching Assistant. 2004. Human Anatomy and Physiology Laboratory, Z 342. Oregon State University.

Teaching Assistant. 2003. Human Anatomy and Physiology Laboratory, Z 341. Oregon State University.

Teaching Assistant. 1997. Introductory Physics Laboratory, PHYS 401. University of New Hampshire.

SELECTED VOLUNTEER POSITIONS & PROFESSIONAL SERVICE

Voting Member. Utah Bird Records Committee. 2011-present.

Trustee. 2009-present. Bridgerland Audubon Society.

Associate Editor. 2007-present. Northwestern Naturalist.

Officer. 2006-2007. Biology Graduate Student Association. Utah State University.

Associate Curator of Amphibians and Reptiles. 2005-2007. Burke Museum, University of Washington.

Landscape and Wildlife Advisory Group. 2004-2006. Cooperative Monitoring, Evaluation, and Research Committee. Washington State Forest Practices Board.

Amphibian and Reptile Science Team. 2005-2006. Landscape Priority Habitats and Species for Developing Areas. Washington Department of Fish and Wildlife.

REVIEWER FOR SCIENTIFIC JOURNALS

Behavioral Ecology and Sociobiology
Forest Science (3 reviews)
Web Ecology
Journal of Herpetology
Caldasia

Al-Rabab'ah MA, Williams CG (2002) Population dynamics of *Pinus taeda* L. based on nuclear microsatellites. For Ecol Manag 163:263-271

Allendorf FW, Leary RF, Spruell P, Wenburg JK (2001) The problem with hybrids: setting conservation quidelines. Trends Ecol Evol 16:613-622

Arnaud-Haond S, Teixeira S, Massa SI, Billot C, Saenger P, Coupland G, Duarte CM, Serrão EA (2006) Genetic structure at range edge: low diversity and high inbreeding in Southeast Asian mangrove (*Avicennia marina*) populations. Mol Ecol 15:3515-3525

Austin JD, Lougheed SC, Boag PT (2004a) Controlling for the effects of history and nonequilibrium conditions in gene flow estimates in northern bullfrog (*Rana catesbeiana*) populations. Genetics 168:1491-1506

Austin JD, Lougheed SC, Boag PT (2004b) Discordant temporal and geographic patterns in maternal lineages of eastern north American frogs, *Rana catesbeiana* (Ranidae) and *Pseudacris crucifer* (Hylidae). Mol Phylogenet Evol 32:799-816

Avise JC (2000) Phylogeography. Harvard University Press, Cambridge

Baxter GT, Stone MD (1985) Amphibians and reptiles of Wyoming, 2nd edn. Wyoming Game and Fish Department, Cheyenne

Biek R, Rodrigo AG, Holley D, Drummond A, Anderson CR, Jr., Ross HA, Poss M (2003) Epidemiology, genetic diversity, and evolution of endemic feline immunodeficiency virus in a population of wild cougars. J Virol 77:9578-9589

Bogan MA, Allen CD, Muldavin EH, Platania SP, Stuart JN, Farley GH, Mehlhop P, Belnap J (1998) Southwest. In: Mac MJ, Opler PA, Puckett Hacker CE, Doran PD (eds) Status and trends of the nation's biological resources. U.S. Department of the Interior, U.S. Geological Survey, Biological Resources Division, Washington, D.C., pp 543-592

Bonnet E, Van de Peer Y (2002) zt: a software tool for simple and partial Mantel tests. J Stat Softw 7:1-12

Bossuyt F, Milinkovitch MC (2000) Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. Proc Natl Acad Sci USA 97:6585-6590

Bradford DF, Tabatabai F, Graber DM (1993) Isolation of remaining populations of the native frog, *Rana muscosa*, by introduced fishes in Sequoia and Kings Canyon National Parks, California. Cons Biol 7:882-888

Brant SV, Ortí G (2003) Phylogeography of the Northern short-tailed shrew, *Blarina brevicauda* (Insectivora: Soricidae): past fragmentation and postglacial recolonization. Mol Ecol 12:1435-1449

Brook BW, O'Grady JJ, Chapman AP, Burgman MA, Akçakaya HR, Frankham R (2000) Predictive accuracy of population viability analysis in conservation biology. Nature 404:385

Caughley G (1994) Directions in conservation biology. J Anim Ecol 63:215-244

Clarkson RW, Rorabaugh JC (1989) Status of leopard frogs (*Rana pipiens* complex: Ranidae) in Arizona and southeastern California. Southwest Nat 34:531-538

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657-1659

Cope ED (1889) The Batrachia of North America. Bull U S Natl Mus 34:1-525

Corn PS, Fogleman JC (1984) Extinction of montane populations of the northern leopard frog (*Rana pipiens*) in Colorado. J Herpetol 18:147-152

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Di Candia MR, Routman EJ (2007) Cytonuclear discordance across a leopard frog contact zone. Mol Phylogenet Evol 45:564-575

Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB (1994) Mutational processes of simple-sequence repeat loci in human populations. Proc Natl Acad Sci USA 91:3166-3170

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Flot J-F (2010) SEQPHASE: a web tool for interconverting PHASE input/output files and FASTA sequence alignments. Mol Ecol Resour 10:162-166

Fontenot BE, Makowsky R, Chippindale PT (2011) Nuclear-mitochondrial discordance and gene flow in a recent radiation of toads. Mol Phylogenet Evol 59:66-80

Frantz AC, Cellina S, Krier A, Schley L, Burke T (2009) Using spatial Bayesian methods to determine the genetic structure of a continuously distributed population: clusters or isolation by distance? J Appl Ecol 46:493-505

Frost JS, Bagnara JT (1976) A new species of leopard frog (*Rana pipiens* complex) from Northwestern Mexico. Copeia 1976:332-338

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Germaine SS, Hays DW (2009) Distribution and postbreeding environmental relationships of Northern Leopard Frogs (*Rana* [*Lithobates*] *pipiens*) in Washington. West N Am Nat 69:537-547

Gibbs EL, Nace GW, Emmons MB (1971) The live frog is almost dead. Bioscience 21:1027-1034

Gibbs JP (1998) Distribution of woodland amphibians along a forest fragmentation gradient. Landscape Ecol 13:263-268

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Goldberg CS, Field KJ, Sredl MJ (2004) Mitochondrial DNA sequences do not support species status of the Ramsey Canyon Leopard Frog (*Rana subaquavocalis*). J Herpetol 38:313-319

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Green DE (2001) Toe-clipping of frogs and toads. In: Standard operating procedure. United States Geological Survey, National Wildlife Health Center

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Harding JH (1997) Amphibians and reptiles of the Great Lakes region. University of Michigan Press, Ann Arbor

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Hine RL, Les BL, Hellmich BF (1981) Leopard frog populations and mortality in Wisconsin, 1974-76. Department of Natural Resources Tech Bull 122:1-39

Hoffman EA, Ardren WR, Blouin MS (2003) Nine polymorphic microsatellite loci for the Northern Leopard Frog (*Rana pipiens*). Mol Ecol Notes 3:115-116

Hoffman EA, Blouin MS (2004a) Evolutionary history of the Northern Leopard Frog: reconstruction of phylogeny, phylogeography, and historical changes in population demography from mitochondrial DNA. Evolution 58:145-159

Hoffman EA, Blouin MS (2004b) Historical data refute recent range contraction as a cause of low genetic diversity in isolated frog populations. Mol Ecol 13:271-276

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Hutchison DW (2003) Testing the central/peripheral model: analyses of microsatellite variability in the eastern collared lizard (*Crotaphytus collaris collaris*). Am Midl Nat 149:148-162

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Kauffeld CF (1937) The status of the leopard frogs, *Rana brachycephala* and *Rana pipiens*. Herpetologica 1:84-87

Kim J-B, Matsui M, Lee J-E, Min M-S, Suh J-H, Yang S-Y (2004) Notes on a discrepency in mitochondrial DNA and allozyme differentiation in a pond frog *Rana nigromaculata*. Zool Sci 21:39-42

Kimberling DN, Ferreira AR, Shuster SM, Keim P (1996) RAPD marker estimation of genetic structure among isolated northern leopard frog populations in the south-western USA. Mol Ecol 5:521-529

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Krueper DJ (1993) Effects of land use practices on western riparian ecosystems. In: Finch DM, Stangel PW (eds) Status and management of neotropical migratory birds. U.S. Department of Agriculture, Forest Service Gen. Tech. Rep. RM-229, Fort Collins, CO, pp 321-330

Lannoo MJ, Lang K, Waltz T, Phillips GS (1994) An altered amphibian assemblage: Dickinson County, Iowa, 70 years after Frank Blanchard's Survey. Am Midl Nat 131:311-319

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Lawson Handley L-J, Estoup A, Evans DM, Thomas CE, Lombaert E, Facon B, Aebi A, Roy HE (2011) Ecological genetics of invasive alien species. BioControl 56:409-428

Lehtinen RM, Galatowitsch SM, Tester JR (1999) Consequences of habitat loss and fragmentation for wetland amphibian assemblages. Wetlands 19:1-12

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    bivalve, *Pinctada maxima*, throughout the Indo-Australian Archipelago shows
    differentiation and decreased diversity at range limits. Mol Ecol 16:5193-5203

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    frequency distributions provides a test for recent population bottlenecks. J Hered
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    habitat fragmentation. Global Ecol Biogeography Lett 1:36-41

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    recent or historic pattern? Biolog Conserv 98:61-68

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    (eds) Amphibians of the Pacific Northwest. Seattle Audubon Society, Seattle, pp
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    populations of the leopard frog, *Rana pipiens*. Evolution 22:274-283

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    Shiozawa DK, Martin AP (2007) Across the great divide: genetic forensics
    reveals misidentification of endangered cutthroat trout populations. Mol Ecol
    16:4445-4454

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    inference of large phylogenetic trees. In: Proceedings of the Gateway Computing
    Environments Workshop (GCE), pp 1-8, New Orleans

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Miller MP, Kimberling DN, Keim P (1999) Re-analysis of genetic structure among populations of *Rana pipiens* in Arizona and Utah. Southwest Nat 44:527-530

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Moriarty ME (2009) Endangered and threatened wildlife and plants; 90-day finding on a petition to list the Northern Leopard Frog (*Lithobates* [=*Rana*] *pipiens*) in the western United States as Threatened. Fed Regist 74:31389-31401

Morris WF, Bloch PL, Hudgens BR, Moyle LC, Stinchcombe JR (2002) Population Viability Analysis in endangered species recovery plans: past use and future improvements. Ecol Appl 12:708-712

Mossman MJ, Hartman LM, Hay R, Sauer JR, Dhuey BJ (1998) Monitoring long-term trends in Wisconsin frog and toad populations. In: Lannoo MJ (ed) Status and conservation of midwestern amphibians. University of Iowa Press, Iowa City, pp 169-198

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Pearson GA, Lago-Leston A, Mota C (2009) Frayed at the edges: selective pressure and adaptive response to abiotic stressors are mismatched in low diversity edge populations. J Ecol 97:450-462

Pimm SL, Dollar L, Bass OL, Jr (2006) The genetic rescue of the Florida panther. Anim Conserv 9:115-122

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Platz JE, Mecham JS (1979) *Rana chiricahuensis*, a new species of leopard frog (*Rana pipiens* complex) from Arizona. Copeia 1979:383-390

Prentis PJ, Wilson JRU, Dormontt EE, Richardson DM, Lowe AJ (2008) Adaptive evolution in invasive species. Trends Plant Sci 13:288-294

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    multilocus genotype data. Genetics 155:945-959

Prychitko TM, Moore WS (1997) The utility of DNA sequences of an intron from the β-

    fibrinogen gene in phylogenetic analysis of woodpeckers (Aves: Picidae). Mol

    Phylogenet Evol 8:193-204

Pujol B, Pannell JR (2008) Reduced responses to selection after species range expansion.

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