AN ABSTRACT OF THE DISSERTATION OF

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A primary goal of population genetics is to identify the role of microevolutionary forces in producing observed patterns of molecular and phenotypic variation. I conducted four studies in the northern leopard frog, *Rana pipiens*, to determine just how mutation, migration, genetic drift, and selection influenced, genetic structure of mitochondrial DNA (mtDNA), nuclear DNA, and a single locus polymorphism that determines dorsal coloration. In the first study, I surveyed the literature concerning color and pattern polymorphisms in anurans. I conclude that anuran polymorphisms remain a rich but largely unexploited system for studying the evolution of phenotypic variation in nature.

In the second study, I compared mitochondrial DNA variation from 35 populations distributed across the species' range. A phylogenetic analysis indicated *R. pipiens* is split into two deeply divergent mtDNA groups, a western group and an eastern group. Phylogeographic and demographic analyses indicated that although restricted gene flow with isolation by distance explained the majority of the processes

influencing current genetic structure. population bottlenecks and expansions also played an important role.

In the third study, I investigated mtDNA and microsatellite variation in Pacific Northwest populations of *R. pipiens*, where a recent range contraction had occurred. I found that peripheral populations had reduced levels of genetic variation compared to more interior populations. Moreover, I found that historic samples from peripheral population already had reduced levels of genetic variation. Therefore, low diversity in the remnant populations could not be ascribed to the recent range contraction.

In the fourth study, I compared genetic structure from a suite of putatively neutral molecular markers with that derived from the color polymorphism locus. Genetic structure at the color locus, assessed both spatially and temporally, was indistinguishable from structure at neutral loci. This study exemplifies the importance of investigating for evidence of selective maintenance before studies attempt to measure the selective mechanisms maintaining a polymorphism.

Overall, my research helps to elucidate how biogeographic and microevolutionary forces influence a wide-spread North American species, *R. pipiens*.

# Natural History and Evolution of a Color Polymorphism in *Rana pipiens*, the Northern Leopard Frog

by

Eric A. Hoffman

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APPROVED:

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Eric A. Hoffman, Author

# ACKNOWLEDGMENTS

This thesis is a work six years in the making and would not have been possible without the help of many people. First, I would like to thank my advisor, Mike Blouin. Without his constant support, open door policy (for any questions – good or bad), extensive library of books for loan, and knowledge of just about every computer program and molecular technique, my projects would not nearly have been so successful. Additionally, I always enjoyed our discussions of the significance of any paper I read, and I have come to learn the importance of being able to know "the take home message" of a paper and be able to write it onto a 3 x 5 notecard. Moreover, our detailed discussions of my data greatly influenced the outcome of this thesis. Mike was always optimistic, especially at the times when I was most discouraged such as when I conducted projects doomed to fail (such as trying to determine the genetic basis for the color polymorphism in *Hyla regilla*) or projects that would have been nearly impossible to complete (such as finding "the green gene"). Mike allowed me to read, come up with my own ideas, and eventually turn those ideas into this thesis. Finally, I am now and have always been grateful for Mike's uncanny ability to return manuscripts (even the poorly written ones) with thoughtful and helpful comments AND usually hand them back within the same week I dumped them onto his desk (and often by the next day).

I would like to thank my committee members Stevan Arnold, Lynne Houck, Phillip McFadden, and George Boehlert (and Dan Rosenberg) for their support and

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During all six of my years at OSU I never once regretted coming to work. This is in large part to who those who shared the Blouin lab with me. I have always had great friends working with me in the lab. Some of my fondest memories at OSU are taking "Blouin lab" day and camping trips into the Cascades of Washington and Oregon to catch frogs. I will never forget my worldly discussions with Wolfe and watching Mike LeMaster clean those sequencing plates. More recently, Anja Bauman was of great assistance with laboratory help and organizational skills. Moreover, three lab members greatly influenced the outcome of my thesis and helped me maintain the sanity to get it finished. Bill Ardren, "the post doc" joined the lab at the end of my graduate school career, just in time to show me how to develop microsatellites and discuss the importance of my work. Also, he, Sarah and Megan have become good family friends. Charles Criscione "mini me" and Kirsten Monsen "the toe ho" are my two academic siblings, and I could not have received better lab mates. Both provided numerous discussions about everything from frog conservation to nested clade analysis to LSU football and Kentucky basketball. Academically, both have discussed all aspects of my data (usually when it was hot off the press) and read all my manuscripts. Equally as important was the non-academic time we all spent hanging

out. I will never forget taking all of Charles' money at poker night or cleaning up the poop from Kirsten's cat every time I watched him. Kirsten I look forward to reading your first manuscript when you analyze the data not using $F_{ST}$, but with $F_{RT}$. Thanks to both of you.

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Most of all I would like to thank my wife, Amanda and my kids, Gabrielle and Will. It is because of them that I had the drive to finish this long endeavor. Amanda has been the best friend I could ask for and just her presence has given me a good

reason to look forward to coming home from work every day. As for the kids, what can I say, nothing gives me more joy than watching them grow and learn.

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# CONTRIBUTION OF AUTHORS

Dr. Michael S. Blouin was directly involved in the design and data analysis of the studies reported in Chapters 2, 3, 4, and 5. Because of his contribution to the research presented in this dissertation, he is a co-author on the four manuscripts submitted for publication resulting from this dissertation. Additionally, Fredrick W. Schueler and Adam G. Jones were directly involved in field work and data anlysis in Chapter 5. Therefore, they are co-authors on the manuscript submitted for publication resulting from this study.

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# DEDICATION

I would like to dedicate my dissertation to my wife, Amanda, and my kids, Gabrielle and Will. Their love and support has given me the resolve to go to work everyday, and the joy of coming home at the end of each day.

Natural History and Evolution of a Color Polymorphism in *Rana pipiens*, the Northern Leopard Frog

# CHAPTER 1: GENERAL INTRODUCTION

Species-wide analyses of population genetic differentiation provide a powerful tool to investigate how the interaction of mutation, migration, genetic drift, and selection have influenced the processes that result in the current population genetic structure of a species. Many studies have used neutral molecular markers to answer questions about a species' phylogeny, phylogeography, and demographic history. These studies have determined inter-specific relationships among species groups, found cryptic species, uncovered patterns of how DNA diversity corresponds with geographic features of the environment, and elucidated the affects of population bottlenecks and expansions. However, these studies seldom address how a trait under the influence of natural selection responds in various populations under different ecological conditions. Moreover, in studies that do indeed discuss a trait under selection, the strength of selection on particular traits has traditionally been inferred from correlations between phenotype and components of fitness within populations (Endler 1986, Kingsolver et al. 2001). By necessity, these sorts of studies have usually been conducted within single populations. The extent to which differences between

populations can be explained by drift and migration, compared to selection, has received less attention.

Species that exhibit visible polymorphisms are ideal for examining the microevolutionary forces that maintain genetic variation in nature. Of these, color or pattern polymorphisms are excellent systems in which to evaluate the relative contribution of drift, migration, and selection because these polymorphisms often have simple Mendelian inheritance, and the phenotypes are easily scored (e.g. Gillespie and Oxford, 1998; Subramaniam and Rausher, 2000). However, most studies of selection on polymorphisms begin with the assumption that inter-populational differences result from selection, and then study the mechanisms by which selection maintains those differences. A more systematic approach would be first to test whether one must infer selection for a set of populations, and *then* proceed to study the details of how selection operates to homogenize or differentiate allele frequencies among populations over evolutionary time.

I investigated the evolutionary history and tested for evidence of selection on a color polymorphism in the northern leopard frog, *Rana pipiens*. *R. pipiens* breeds during the spring, and tadpoles metamorphose from early summer to early fall. By metamorphosis, tadpoles exhibit a conspicuous polymorphism in which an individual develops either green or brown permanent coloration on its dorsum. This striking color polymorphism is controlled by a one locus, two-allele system in which the green allele is dominant to the brown allele (Fogleman et al. 1976). Thus, the easily scored phenotypic frequencies can be directly translated into

genotypic frequencies, assuming populations are in Hardy-Weinberg Equilibrium (HWE).

The leopard frog species complex has a storied history in evolutionary biology (see Hillis 1988). Various aspects of *R. pipiens* ecology have been well studied (Moore 1949; Dole 1971; Merrell 1977), but relatively few studies have looked at genetic variation within *Rana pipiens sensu stricto*. The northern leopard frog is broadly distributed, with a species range from the state of Washington to New York, and from Arizona to Hudson Bay. The majority of previous studies investigating geographic variation within leopard frogs have not differentiated among species within the complex (Moore 1949; Gillespie and Crenshaw 1966; Post and Pettus 1966; Salthe 1969), or only used limited sampling of northern leopard frogs (Dunlap and Platz 1981; Kimberling et al. 1996) and therefore provide little information about species-wide phylogeography. Moreover, *R. pipiens* is an ideal species for investigating how selection acts on the color polymorphism for several additional reasons. First, the visually conspicuous green/brown polymorphism allows for easy scoring of phenotypic frequencies (pers. obs). Second, *R. pipiens* is widespread with a geographic range spanning much of North America (Conant and Collins, 1991). Third, the polymorphism exists over the entire species range. These circumstances enable an investigation of how evolutionary forces act on the polymorphism under a variety of environmental conditions.

Therefore, I undertook a systematic approach to understanding how migration, genetic drift and selection influence the current distribution of the northern leopard frog at both neutral loci and a color polymorphism locus putatively influenced by natural selection. In Chapter 2, I conducted a literature review to elucidate evolutionary patterns from the large number of anuran color polymorphisms for which data had been collected. This study focused on five main topics: proximate basis of color or pattern variation, color change, sexual dimorphism, modes of inheritance, and evidence for natural selection. My goal in this study was to identify major gaps in the understanding of anuran polymorphisms, and to highlight potential systems for future work on the evolution and maintenance of these polymorphisms.

In Chapter 3, I investigated the evolutionary history of the northern leopard frog. This study uses a combined methodological approach including phylogenetic, phylogeographic, and demographic analyses to understand the evolutionary history of the northern leopard frog. Using these analyses I tested hypotheses concerning how (or if) known geological events and key features of the species biology influenced the contemporary geographic and genetic distribution of *R. pipiens*. I assayed mitochondrial DNA variation from 389 individuals within 35 populations located throughout the species range to test predictions concerning the phylogeographic and demographic history of *R. pipiens*. First, I predicted that restricted gene flow plays an important role in the contemporary genetic composition of *R. pipiens* because studies have shown that isolation by distance

and restricted movement are the main factors that influence anuran genetic structure (Yang et al. 1994; Driscoll 1998; Shaffer et al. 2000; Nielson et al. 2001; Rowe et al. 2000, Newman and Squire 2001; Monsen and Blouin, unpublished data). Second, I predicted that, within the framework of restricted gene flow, I would be able to detect the genetic signature of a demographic bottleneck and subsequent expansion caused by glacial advance and retreat (Hewitt 1996), including evidence of glacial refugia (Comps et al. 2001). These a priori hypotheses arose from two sources; (1) phylogeographic and population genetic studies of anurans in general and (2) phylogeographic studies of other taxa within temperate North America.

One interesting finding from Chapter 3 was that Pacific Northwest populations of the northern leopard frog contained unique mtDNA haplotypes. This uniqueness is interesting because these populations are in a region where amphibians in general have been declining (Leonard et al. 1999). In Chapter 4, I investigated how these known population bottlenecks contributed to molecular marker variation within peripheral and interior Pacific Northwest populations. In this study I tested whether low genetic diversity in remnant populations of Pacific Northwest northern leopard frogs is best explained by a recent bottleneck or by a history of being peripheral. First, I assayed mitochondrial DNA (mtDNA) variation to verify that the putative remnant populations were actually native remnants, and not introduced populations from laboratory stock or the pet trade. Second, I used eight microsatellite loci to compare genetic diversity in remnants

with diversity in historical samples from the same region (i.e. the former periphery of the species range) that were collected before the population decline. I also compared genetic diversity in historical and extant interior populations. I conclude by discussing the implications of my results for management of the evolutionary legacy of *R. pipiens* and of peripheral populations in general.

In Chapter 5, I analyzed the population genetic structure for the color locus, a locus putatively maintained by selection. Specifically, I took a locus comparison approach to identify different genetic patterns between putatively neutral loci (from molecular markers) and the color locus. First, I used both a large- and small-scale spatial approach to determine if the color locus exhibited a pattern of genetic variation among populations that is different from that at neutral loci. At a large spatial scale, one might expect genetic variation among populations at the color locus to be less than that observed at neutral loci (smaller $F_{ST}$) owing to stabilizing selection on the color locus. Across a small spatial scale, one might expect either higher or lower $F_{ST}$, depending on whether the selectively maintained equilibrium allele frequencies are the same or different among populations. Second, I used temporally spaced samples to determine if observed allele frequency change through time at the color locus is different than change through time at a simulated neutral bi-allelic locus. Finally, I used computer simulations to determine the relative values of the selection coefficient (assuming frequency-dependent selection or overdominance) necessary to be detected given my techniques. I then discuss

the impact of my results for future studies testing the selective maintenance of polymorphism.

# CHAPTER 2.
# A REVIEW OF COLOR AND PATTERN POLYMORPHISMS IN ANURANS

ABSTRACT

Species that exhibit polymorphism, the simultaneous occurrence of two or more discrete, genetically-based phenotypes in a population, are ideal for studying the microevolutionary forces that maintain genetic variation in nature. Many anuran species exhibit striking color or dorsal pattern polymorphisms, and so provide an excellent system in which to study questions pertaining to the evolution and maintenance of polymorphisms. Despite the wide occurrence of pattern or color polymorphisms in anurans (current records cite at least 225 species representing 35 genera and 11 families) surprisingly little conclusive work has been done on the inheritance and selective maintenance of this variation. The mode of inheritance has been investigated in 26 species, but conclusively demonstrated in only two. Fourty-six species have been described as undergoing ontogenetic change, and thirty-two species have been described as sexually dimorphic. That anuran polymorphisms are under some sort of selection has been inferred from the large number of polymorphic species, from putative cases of apparent convergent evolution and the existence of identical polymorphisms in closely related species, from cyclical fluctuations in morph frequencies, and from a few observations of non-random survival during bouts of stress. The selective mechanisms maintaining these polymorphisms have been investigated in only 19 species. Most studies looked for physiological traits correlated with the polymorphism, rather than studying the most obvious mechanism, selective predation on color/pattern itself.

Thus, anuran polymorphisms remain a rich but largely unexploited system for studying the evolution of phenotypic variation in nature.

## INTRODUCTION

Polymorphism can be defined as the simultaneous occurrence of two or more discrete, genetically-based phenotypes in a population, in which the frequency of the rarest type is higher than can be maintained by recurrent mutation (Ford, 1975; e.g. greater than 1% given reasonable values for selection and mutation rates in finite populations; Hartl & Clark, 1997; Hedrick, 1985). Species that exhibit visible polymorphisms are ideal for examining the microevolutionary forces that maintain genetic variation in nature. This is because phenotypes are easily scored, and because color or pattern polymorphisms often have simple Mendelian inheritance. Classic examples include studies of the pepper moth, *Biston betalaria* (Kettelwell, 1961), and the land snail, *Cepaea* sp. (Jones, Leith, & Rawlings, 1977).

Many anuran species exhibit striking color or dorsal pattern polymorphisms, and so provide an excellent system in which to study questions pertaining to the evolution and maintenance of polymorphisms. Nevertheless, surprisingly little conclusive work has been done on the inheritance and selective maintenance of anuran polymorphisms, so the system remains largely unexploited. Here I review the literature on anuran color or pattern (hereafter CP) polymorphisms by focusing on five main topics: proximate basis of color or pattern variation, color change, sexual dimorphism, modes of inheritance, and evidence for natural selection. My goals in this review are to identify major gaps in the

understanding of anuran polymorphisms, and to highlight the potential of this system for future work on the evolution and maintenance of genetic variation in nature.

## TYPES OF VARIATION AND TAXONOMIC DISTRIBUTION

Published accounts describe discrete CP polymorphisms in 11/23 (48%) anuran families (Table 2.1). Thirty-five genera are represented within these 11 families, and at least 225 different species are known to be polymorphic. Interestingly, over one third of these are *Eleutherodactylus* species, and *Phrynobatrachus* and *Hyperolius* represent one fourth of the species (Table 2.2). All three of these genera are extremely speciose, and *Eleutherodactylus* and *Phrynobatrachus* are poorly studied. Anuran polymorphisms manifest as variation in body color or in dorsal pattern. The presence or absence of dark stripes or spots on the dorsum is a common polymorphism in many families of anurans (Table 2.1). Color variation can involve the entire body, or just parts of the dorsum. Polymorphism for red, green or brown/gray dorsal color is a common motif. Some species exhibit a combination of the above designs; for example, variation in *Hyla regilla* includes presence/absence of dorsal stripes as well as an array of body colors (Resnick & Jameson, 1963).

Despite the abundance of polymorphic species, surprisingly little is actually known about these polymorphisms. For most species, all we have are brief

descriptions or anecdotal observations of a polymorphism (e.g. most *Eleutherodactylus* species, as well as the species listed in Laurent, 1950 & 1957; Glaw & Vences, 1992; Stewart, 1974; Duellman & Crump, 1974; Jones, 1963; Savage, 1966; Karlstrom, 1973; Martoff & Humphries, 1959; Edwards, 1974). As I will illustrate below, conclusive data on modes of inheritance are available for only a handful of species, and studies on the selective maintenance of anuran polymorphisms are still in their infancy. I begin by reviewing the proximate physiological and morphological causes of CP variation.

## PROXIMATE BASIS OF THE PHENOTYPIC VARIATION

Color and pattern in anurans is primarily controlled by two cell types: melanophores, which contain melanin, and chromatophores, which contain colored pigments. The melanin within melanophores is contained in organelles called melanosomes. Melanophores lie below the chromatophores, but have extensive cellular processes that extend up and over the chromatophores. When the skin appears light in color, the melanosomes within the melanophore are concentrated in the cell body. When the skin darkens, the melanosomes spread out into the processes of the melanophore covering the chromatophores, thus obscuring the bright colors (Frost-Mason, Morrison, & Mason, 1994). Factors that affect movement of the pigment include temperature, light, and humidity. Warm and dry conditions result in contraction of the pigment granules, making the frog pale; cold

and damp conditions facilitate the dispersal of pigment, making the frog appear darker. Because a dark-colored frog will lose heat quicker when its temperature is higher than the surroundings, or absorb more heat when exposed to the sun than will a light-colored frog, some frogs thermoregulate via changing color (Passmore and Carruthers, 1979). Dispersal of the pigment granules is controlled by the pituitary gland, which produces melanophore-stimulating hormone (MSH) in response to various stimuli, including visual cues (Bagnara, 1976; Heatwole, 1994). In the case of *Rana pipiens*, spots or stripes are created by increased concentrations of melanin per melanophore, rather than by an increase in number of melanophores per unit area (Baker, 1951). Similarly, the pale morph in *Bombina orientalis* results from a reduction in melanin concentration per melanophore, rather than from a reduced density of melanophores (Ellinger, 1980). Nevertheless, not all pale and/or dark variants are caused directly by increasing or decreasing melanin concentration. Dark variants of *Ptychadena mascrareniensis*, *Rana clamitans*, and *R. pipiens* primarily result from a lack of iridophores, which are a type of chromatophore (Richards & Nace, 1983).

Chromatophores are divided into two main categories: non-reflecting and reflecting. Non-reflecting chromatophores that contain carotenoids, pteridines, and flavins are called xanthophores when predominantly yellow, and erythrophores when predominantly red. Reflecting chromatophores that contain the purines guanine, hypoxanthine, and adenine are called iridophores (Bagnara, 1966; Heatwole, 1994). These cell types work together to cause the colors found in

Table 2.1. Species for which there is a record of color or pattern variation. Species are categorized into families as listed by Frost (1985).

| Family | Species | Type of Polymorphism | Reference |
|---|---|---|---|
| Arthroleptidae | *Arthroleptis adolfifriederici* Loveridge | Dorsal color variation: gray, tan, or green | Stewart, 1967 |
| Bufonidae | *Bufo arenarium* Hensel | Presence/absence of yellow spots | Cei, 1959 |
| | *Bufo canorus* Camp | Females: black spots on gray or brown. Males: uniform yellow-green to olive | Karlstrom, 1973 |
| | *Bufo houstonensis* Sanders | Ontogenetic color change from beige to white | Mays & Freed, 1985 |
| | *Bufo periglenes* Savage | Females are olive/black; Males are orange | Savage, 1966 |
| | *Bufo raddei* Strauch | Sexual dimorphism noted, but not described | Savage, 1966 |
| | *Bufo regularis* Reuss | Presence/absence of vertebral stripe | Stewart, 1967 |

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| | *Bufo spinulosus* Wiegmann | Presence/absence of yellow spots | Cei, 1959 |
| | *Bufo taitanus* Peters | Males: middorsal pattern of 3 dark blotches. Females: light reddish-brown dorsum with dark flanks | Stewart, 1967 |
| | *Bufo typhonius* Linnaeus | Body pattern and color variation | Jones, 1963 |
| Dendrobatidae | *Colostethus pulchellus* Jimenez de la Espada | Dorsal stripe variations | Edwards, 1974 |
| | *Dendrobates histrionicus* Berthold | Extremely variable in color pattern. | Myers & Daly, 1976 |
| | *Dendrobates leucomelas* Steindachner | Born with yellow bands around waist, black spots develop within yellow band as they mature. | Mattison, 1998 |
| | *Dendrobates pumilio* O. Schmidt | Dorsal pattern variation | Myers & Daly, 1983 |
| Discoglossidae | *Bombina orientalis* | Rare pale morph | Ellinger, 1980 |

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|---|---|---|---|
| | Boulenger | | |
| | *Discoglossus pictus* Otth | Stripe and spot pattern variation | Bruce & Parkes, 1947; Lantz, 1947 |
| Hylidae | *Acris crepitans* Baird, *Acris gryllus* LeConte | Gray, green, or red vertebral stripe | Pyburn, 1961a; Pyburn, 1961b; Wendelken, 1968; Isaacs, 1971; Gray, 1972; Nevo, 1973; Gray, 1977; Gray, 1978; Gray, 1983; Gorman, 1986 |
| | *Agalychnis callidryas* Cope | Development of spotting pattern | Starret, 1960; Duellman, 1983 |
| | *Amphignathodon guentheri* Boulenger | Tan, dark brown, or uniform green | Duellman & Ruiz-C, 1986 |
| | *Gastrotheca andaquiensis* Ruiz & Hernandez | Males are brown and females are green | Lynch, pers. comm. |
| | *Gastrotheca aureomaculata* Cochran & Goin | Brown to black with gold spots, or green with no spots | Duellman & Ruiz-C, 1986; Duellman, 1983; Titus, Hillis, & Duellman, 1989 |

| | | |
|---|---|---|
| *Gastrotheca griswoldi* Shreve | Young juveniles are green, old juveniles are brown to gray. | Duellman & Ruiz-C, 1986 |
| *Gastrotheca helenae* Dunn | Juveniles are always light tan, adults are tan to dark brown. | Duellman & Ruiz-C, 1986 |
| *Gastrotheca peruana* Boulenger | Dorsal pattern variation | Duellman & Fritts, 1972 |
| *Hyla bokermanni* Goin | Female: 2 dorso-lateral stripes, males: diffusely spotted or speckled | Rivero, 1969 |
| *Hyla leucophyllata* Beireis | Color pattern, variation not described | Titus et al., 1989 |
| *Hyla luteocellata* Roux | Female: 2 dorso-lateral stripes, males: diffusely spotted or uniform | Rivero, 1969 |
| *Hyla parviceps* group Boulenger | Variation noted but not described | Duellman & Crump, 1974; Rivero, 1969; Duellman & Trueb, 1989 |
| *Hyla regilla* Baird and Girard | Red, green, brown, or gray color morphs | Test, 1898; Brattstrom & |

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|---|---|---|
| | | Warren, 1955; Resnick & Jameson, 1963; Jameson & Pequegnat, 1971; Schuab & Larson, 1978; Morey, 1990 |
| *Hyla triangulum* Gunther | Dorsal spotting variation | Duellman, 1974 |
| *Phrynohyas venulosa* Laurenti | Dorsal striping variation | Duellman, 1971 |
| *Pseudacris ornata* Holbrook | Brown, red, green, and green spot color morphs | Travis & Trexler, 1984; Harkey & Semlitsch, 1988; Blouin, 1989a; Blouin, 1989b |
| *Pseudacris triseriata* Wied-Neuwied | Brown, gray, red, or green color morphs | Mathews & Pettus, 1966; Tordoff & Pettus, 1977; Mathews, 1971; Tordoff, 1980; Hoppe & Pettus, 1984 |

| | | | |
|---|---|---|---|
| | | Albino morphs in high frequency | Corn, 1986 |
| | *Smilisca baudinii* Dumeril & Bibroon | Variation noted but not described | Duellman & Trueb, 1966 |
| Hyperoliidae | *Afrixalus fornasini* Bianconi | Presence/absence of middorsal stripe | Schiotz, 1975 |
| | *Afrixalus fulvovittatus* Peters | Juveniles have uniform dark coloration, adults develop dorsal and lateral stripes | Laurent, 1950 |
| | *Afrixalus orophilus* Laurent | Juveniles have uniform dark coloration, adults develop dorsal and lateral stripes | Laurent, 1950 |
| | *Heterixalus variabilis* Ahl | Males are without conspicuous coloration, females are beige to yellow with large black markings | Glaw & Vences, 1992 |
| | *Hyperolius alborufus* Laurent | Dorsal pattern polymorphism | Schiotz, 1971 |
| | *Hyperolius* | Dorsal pattern | Schiotz, 1971 |

| | | |
|---|---|---|
| *argentovittis* Ahl | polymorphism | |
| *Hyperolius argus* Peters | Males and juveniles: pale green. Females: pale to dark brown with white spots. | Stewart, 1967, Schiotz, 1975; Hayes, 1997, Hayes & Menendez, 1999 |
| *Hyperolius castanus* Ahl | Juveniles have white dorso-lateral stripe that is generally retained in males, but not so in females. Males and females possess many patterns of stripes and spots | Laurent, 1950 |
| *Hyperolius cinnamomeoventris* Bocage | Males and juveniles: brown or green with dorsolateral stripe. Females: uniform green | Laurent, 1950; Laurent, 1957; Schiotz, 1975 |
| *Hyperolius cystocandicans* Richards & Schiotz | Juveniles are pale green with gold/white stripe, adults are continuous to mottled with yellow, orange or brown | Richards & Schiotz, 1977 |

| | | |
|---|---|---|
| *Hyperolius discodactylus* Ahl | White stripe on sides and back of males, not present in females | Laurent, 1950 |
| *Hyperolius glandicolor* Peters | Juveniles are hourglass patterned, females are dark dorsum with white spots | Schiotz, 1971 |
| *Hyperolius granulatus* Boulenger | Juveniles four stripes on dorsum that fade with age (no stripes in adults) | Laurent, 1957 |
| *Hyperolius huillensis* Bocage | Dorsal pattern polymorphism | Schiotz, 1971 |
| *Hyperolius karisimbiensis* Ahl | Juveniles have dorsal pattern that completely disappears in females and disappears in some males | Laurent, 1950 |
| *Hyperolius kibarae* Laurent | Juveniles have dorso-lateral stripes that fade with age (no stripes in adults) | Laurent, 1957 |
| *Hyperolius kivuensis* Ahl | Juveniles have three dorso-lateral stripes. | Laurent, 1950 Laurent, 1957; |

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|---|---|---|
| | These stripes are sometimes retained in adult males and seldom retained in adult females. Also, green to brown ontogenetic change | Schiotz, 1975 |
| *Hyperolius lateralis* Laurent | Juveniles are green, females are dark green with a broad cream lateral stripe | Schiotz, 1975 |
| *Hyperolius langi* Noble | Two discrete pattern groups in males, too few females have been investigated to see if same polymorphism exists | Laurent, 1950 |
| *Hyperolius mariae* Barbour & Loveridge | Juveniles are hourglass patterned, females are uniform yellow or brown | Schiotz, 1971 |
| *Hyperolius marmoratus* Rapp | Juveniles are brown with hourglass pattern, females are uniform yellow or brown with four dark | Stewart, 1967; Passmore & Carruthers, 1979; Mattison, 1998 |

| | | |
|---|---|---|
| | stripes | |
| *Hyperolius mitchelli* Loveridge | Juveniles are brown with dark spots, females are brown with white dorsolateral stripe | Schiotz, 1975 |
| *Hyperolius nasutus* Gunther | Juveniles and some males have dorso-median and doro-lateral stripes. Other males and all females lack all three stripes. | Laurent, 1950 |
| *Hyperolius nitidulus aureus* Perret | Presence/absence of dorsal spots | Schiotz, 1971 |
| *Hyperolius nyassae* Ahl | Juveniles are hourglass patterned, females are uniform yellow with black spots | Schiotz, 1971 |
| *Hyperolius ocelatus* Ahl | Females have small black spots and males do not. | Laurent, 1950 |
| *Hyperolius parallelus* Gunther | Juveniles are hourglass patterned, females are dark with middorsal stripe | Schiotz, 1975 |
| *Hyperolius pictus* | Juveniles are brown with | Stewart, 1967 |

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| Ahl | three green stripes, adults are green & brown with stripes and spots | |
| *Hyperolius platyceps* Boulenger | Juveniles have hourglass pattern, adults have dorso-lateral stripes | Laurent, 1957 |
| *Hyperolius pleurospilus* Laurent | Males and females differ in coloration, but not described | Laurent, 1950 |
| *Hyperolius pusillus* Cope | Presence/absence of black dorsal stripes and spots | Passmore & Carruthers, 1979 |
| *Hyperolius puncticulatus* Pfeffer | Juveniles are brown with diffuse mottling, females are red-brown with spots | Schiotz, 1975 |
| *Hyperolius quinquevittatus Bocage* | Juveniles have longitudinal black stripes. In adult, males have golden/brown dorsum with stripes, females have green dorsum with lateral stripes | Laurent, 1957; Schiotz, 1975 |
| *Hyperolius* | Juveniles are brown with | Schiotz, 1975 |

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|---|---|---|
| *rubrovermiculatus* Schiotz | dark spots, females are dark with red spots | |
| *Hyperolius spatzi* Ahl | Juveniles are white, females are light gray with black spots | Schiotz, 1971 |
| *Hyperolius spingularis* Stevens | Pattern polymorphism: light triangle on snout or dorsolateral stripes | Schiotz, 1975 |
| *Hyperolius tuberculatus* Laurent | Juveniles are hourglass patterned, females are gray with black spots | Schiotz, 1971 |
| *Hyperolius tuberilinguis* Smith | Juveniles have brown or mottled green dorsum, females are bright green | Stewart, 1967; Passmore & Carruthers, 1979 |
| *Hyperolius v. viridiflavus* Dumeril & Bibroon | Juveniles are brown, females are green, many pattern variations | Laurent, 1950; Schiotz, 1971; Richards, 1976; Richards, 1982 |
| *Leptopelis natalensis* Smith | Dorsal pattern polymorphism | Passmore & Carruthers, 1979 |

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|---|---|---|---|
| | *Leptopelis concolor* Poynton | Juveniles are green, females are tan to dark brown | Passmore & Carruthers, 1979 |
| | *Leptopolis flavomaculatus* Gunther | Green/brown color polymorphism | Stewart, 1967; Schiotz, 1975 |
| | *Leptopolis vermiculatus* Boulenger | Green/brown color polymorphism | Schiotz, 1975 |
| | *Taychynemis seychellensis* Dumeril & Bibron | In some populations males are brown, females are green, | Nussbaum & Wu 1995 |
| Leptodactylidae | *Eleutherodactylus* sp. Dumeril & Bibroon | Dorsal stripe pattern variation | See Table 2. |
| Microhylidae | *Breviceps poweri* Parker | Presence/absence of vertebral stripe | Stewart, 1967 |
| | *Cophyla phyllodactyla* Boettger | Presence/absence of beige vertebral stripe | Glaw & Vences, 1992 |
| | *Scaphiophryne* | Dorsum is light brown or | Glaw & Vences, |

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|---|---|---|---|
| | *calcarata* Mocquard | light green | 1992 |
| Myobatrachidae | *Crinia georgiana* Tschudi, *C. glauerti* Loveridge | Dorsal pattern variation | Main, 1965; Main, 1968; Bull, 1975 |
| | *Crinia insignifera* Moore, *C. pseudoinsignifera* Main | Dorsal pattern variation | Main, 1961; Main, 1965; Main, 1968; Bull, 1975; Bull, 1977 |
| | *C. (Ranidella) parinsignifera* Main, *C. signifera* Girard, *C. subinsignifera* Littlejohn, *C. tinnula* Straughan & Main | Dorsal pattern variation | Bull, 1975 |
| Ranidae | *Cacosternum boettgeri* Boulenger | Green/brown color polymorphism | Passmore & Carruthers, 1979 |
| | *Hildebrantia* sp. Nieden | Pattern polymorphism – variation not described | Stewart, 1974 |
| | *Limnonectes tigerinus* Daudin | Green/brown color polymorphism | Glaw & Vences, 1992 |

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|---|---|---|
| *Mantella aurantiaca* Mocquard | Uniform yellow, orange, or red | Glaw & Vences, 1992 |
| *Mantella crocea* Pintak & Bohme | Yellow/orange/green color polymorphism, and presence/absence of reticulation | Glaw & Vences, 1992 |
| *Mantidactylus aerumnalis* Peracca | Green/brown color polymorphism and presence/absence of light vertebral stripe | Glaw & Vences, 1992 |
| *Mantidactylus depressiceps* Boulenger | Dorsum is uniform gray, brown, or black | Glaw & Vences, 1992 |
| *Mantidactylus granulatus* Boettger | Presence/absence of dark spots on dorsum | Glaw & Vences, 1992 |
| *Mantidactylus guttulatas* Boulenger | Presence/absence of small yellow spots on dorsum | Glaw & Vences, 1992 |
| *Mantidactylus liber* Peracca | Red/green/gray color polymorphism and presence/absence of light vertebral stripe | Glaw & Vences, 1992 |

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|---|---|---|
| *Mantidactylus lugubris* Dumeril | Brown, black, or green coloration | Glaw & Vences, 1992 |
| *Mantidactylus peraccae* Boulenger | Green/brown color polymorphism | Glaw & Vences, 1992 |

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| *Phrynobatrachus accraensis* Ahl, *P. acridoides* Cope, *P. cryptotis* Schmidt & Inger, *P. francisci* Boulenger, *P. graueri* Nieden, *P. keniensis* Barbour & Loveridge, *P. minutus* Boulenger, *P. natalensis* Smith, *P. ogoensis* Boulenger, *P. parkeri* Witte, *P. parvulus* Boulenger, *P. tokba* Chabanaud, *P. ukingensis* Loverridge, *P. werneri* Nieden | Dorsal pattern variation and presence/absence of dorsal stripe | Stewart, 1967; Stewart, 1974 |
| *Phrynobatrachus alleni* Parker, *P. bequaerti* Barbour | Presence/absence of vertebral stripe | Stewart, 1974 |

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| & Loveridge, *P. calcaratus* Peters, *P. cricogaster* Perret, *P. dendrobates* Boulenger, *P. fraterculus* Chabanaud, *P. kinangopensis* Angel, *P. parogoensis*, *P. perpalmatus* Boulenger, *P. petropeditoides* Ahl, *P. rungwensis* Loverridge, *P. versicolor* Ahl | | |
| *Ptychadena mascareniensis* Dumeril & Bibron | Dark pigment variant | Richards & Nace, 1983 |
| | Green/brown color polymorphism and presence/absence of | Glaw & Vences, 1992 |

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|---|---|---|
| | vertebral stripe | |
| *Pyxicephalus* sp. Tschudi | Pattern polymorphism – variation not described | Stewart, 1974 |
| *Rana arvalis* Nilsson | Presence/absence of dorsal stripe | Ishchenko & Shchupak, 1974, Ishchenko, 1994 |
| *Rana clamitans* Latreille | Rare blue variant | Berns & Narayan, 1970 |
| | Dark pigment variant | Richards & Nace, 1983 |
| *Rana limnocharis* Boie | Presence/absence of yellow dorso-median stripe | Moriwaki, 1953 |
| *Rana macrocnemis* Boulenger | Variation noted but not described | Ishchencko, 1994 |
| *Rana nigromaculata* Hallowell | Presence/absence of dorso-median stripe and ventral markings | Moriya, 1952 |
| *Rana pipiens* Schreber | Spot pattern variation | Browder & Davison, 1964; Merrell & Rodell, 1968; Moore, 1942; Moore, 1943; |

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| | | | Volpe, 1955; Volpe, 1956; Volpe, 1960; Volpe, 1961a; Volpe, 1961b; Volpe and Dasgupta, 1962a; Volpe and Dasgupta, 1962b; Volpe, 1963; Anderson & Volpe, 1958; Davison, 1961; McKinnell, 1962; Davison, 1963; Davison, 1964; Merrell, 1965; Merrell, 1972; Underhill, 1968; Gill and Nace, 1969; Volpe, 1970; Gill, 1970; Volpe, 1970; Schueller 1979 |
| | | Green and Brown color morphs | Fogleman et al., 1980; Corn, 1982 |
| | | Dark pigment | Richards & Nace, |

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|---|---|---|---|
| | | variant/Albanism | 1983; Browder, 1972 |
| | *Rana septentrionalis* Baird | Color (tan – green) and dorsal spotting variation | Kramek & Stewart, 1980 |
| | *Rana sphenocephala* Cope | Presence/absence of spotting pattern | Brown & Funk, 1977 |
| | *Rana sylvatica* Leconte | Presence/absence of white dorsal stripe | Martof & Humphries, 1959; Browder et al., 1966; Fishbeck & Underhill, 1971; Schueler & Cook, 1980 |
| | | Dark pigment variant | Richards & Nace, 1983 |
| Rhacophoridae | *Aglyptodactylus madagascariensis* Dumeril | Presence/absence of vertebral stripe | Glaw & Vences, 1992 |
| | *Boophis mandrake* Bloomers-Schlosser | Presence/absence of large yellow spots on dorsum | Glaw & Vences, 1992 |
| | *Boophis microtympanum* Boettger | Males are green, females are yellow-green | Glaw & Vences, 1992 |

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|---|---|---|---|
| | *Boophis pauliani* Guibe | Males are green, females are brown | Glaw & Vences, 1992 |
| | *Boophis tephraeomystax* Dumeril | Presence/absence of large brown spots and markings | Glaw & Vences, 1992 |
| | *Polypedates (Rhacophorus) leucomystax* Gravenhorst | Brown, green, gray color morphs and spotted or striped pattern | Church, 1963 |

Table 2.2: *Eleutherodactylus* sp. exhibiting pattern polymorphism

| Species | Reference |
|---|---|
| *E. achatinus* | Lynch & Duellman, 1997 |
| *E. actinolaimus* | Lynch & Rueda, 1998a |
| *E. affinis* | Cochran & Goin, 1970 |
| *E. agusti* | Mattison, 1998 |
| *E. alticola* | Lynn, 1940 |
| *E. amadeus* | Hedges, Thomas, & Franz, 1987 |
| *E. antillensis* | Schmidt, 1920; Schmidt, 1928 |
| *E. apiculatus* | Lynch & Duellman, 1997 |
| *E. auriculatus* | Schmidt, 1920 |
| *E. bicolor* | Rueda & Lynch, 1993 |
| *E. bicumulus* | Lynch & LaMarca, 1993 |
| *E. binotatus* | Heyer et al., 1990 |
| *E. biporcatus* | A. Crawford, pers. comm. |
| *E. bogotensis* | Cochran & Goin, 1970 |
| *E. bransfordii* | Savage & Emerson, 1970 |
| *E. brevifrons* | Lynch, 1998 |
| *E. brittoni* | Schmidt, 1928 |
| *E. calcaratus* | Cochran & Goin, 1970 |

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| *E. carmelitae* | Cochran & Goin, 1970 |
| *E. carvalhoi* | Rodríguez & Duellman, 1994 |
| *E. caryophyllaceus* | A. Crawford, pers. comm. |
| *E. colodactylus* | Lynch, 1979 |
| *E. cundalli* | Lynn, 1940 |
| *E. elassodiscus* | Lynch, 1973 |
| *E. erythropleura* | Lynch, 1992 |
| *E. factiosus* | Lynch & Rueda, 1998a |
| *E. fitzingeri* | A. Crawford, pers. comm. |
| *E. gossei* | Goin, 1954 |
| *E. griphus* | Crombie, 1986 |
| *E. gryllus* | Schmidt, 1928 |
| *E. guentheri* | Heyer et al., 1990 |
| *E. gularis* | Cochran & Goin, 1970 |
| *E. hamiotae* | Flores, 1993 |
| *E. junori* | Goin, 1954 |
| *E. laticeps* | Savage, 1987 |
| *E. laticlavius* | Lynch & Duellman, 1997 |
| *E. latidiscus* | Lynch & Duellman, 1997 |
| *E. lauraster* | Savage, McCranie, & Espinal, 1996 |
| *E. llojsintuta* | Kohler & Lotters, 1999 |

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| *E. luteolus* | Lynn, 1940 |
| *E. megalops* | Lynch & Ruíz-Carranza, 1985 |
| *E. mexicanus* | Lynch, 1966 |
| *E. nasutus* | Goin, 1950 |
| *E. nasutus* | Lynn & Lutz, 1947 |
| *E. nubicola* | Goin, 1950; Lynn, 1940 |
| *E. nubicola* | Goin, 1960 |
| *E. nyctophylax* | Lynch & Duellman, 1997 |
| *E. ockendeni* | Lynch, 1974a |
| *E. orcutti* | Lynn, 1940; Goin, 1950 |
| *E. orpacobates* | Lynch, Ruiz, & Ardila, 1994 |
| *E. pantoni* | Goin, 1950; Lynn, 1940 |
| *E. parvillus* | Lynch & Duellman, 1997 |
| *E. parvus* | Heyer et al., 1990 |
| *E. permixtus* | Lynch, Ruíz-Carranza, & Ardila-Robayo, 1994 |
| *E. phoxocephalus* | Lynch, 1979; Lynch & Duellman, 1997 |
| *E. podiciferus* | Lynch, 1965 |
| *E. polychrus* | Ruiz, Lynch, & Ardila, 1996 |
| *E. portoricensis* | Schmidt, 1928 |
| *E. proserpens* | Lynch, 1979 |
| *E. pygmaeus* | Lynch, 1966 |

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|---|---|
| *E. pyrrhomerus* | Lynch & Duellman, 1997 |
| *E. randorum* | Heyer et al., 1990 |
| *E. rhodopis* | Lynch, 1966 |
| *E. ricordii* | Goin, 1947; Lynn, 1940 |
| *E. ridens* | A. Crawford, pers. comm. |
| *E. riveti* | Lynch, 1979 |
| *E. rosadoi* | Lynch & Duellman, 1997 |
| *E. sanguineus* | Lynch, 1998 |
| *E. sanctaemartae* | Lynch & Ruíz-Carranza, 1985 |
| *E. stejnegerianus* | Scott, 1983; A. Crawford pers. comm. |
| *E. suetus* | Lynch & Rueda, 1998b |
| *E. taeniatus* | Lynch, 1980 |
| *E. tayrona* | Lynch & Ruiz, 1985 |
| *E. uranobates* | Lynch, 1995 |
| *E. versicolor* | Lynch, 1979 |
| *E. vertebralis* | Lynch & Duellman, 1997 |
| *E. vidua* | Lynch, 1979 |
| *E. vocator* | A. Crawford, pers. comm. |
| *E. walkeri* | Lynch, 1974b |
| *E. w-nigrum* | Lynch & Duellman, 1997 |

anuran skin. For example, green in at least two anurans (*Hyla cinerea* and *R. pipiens*) is not caused by a green pigment (Lyerla & Jameson, 1968; Browder, 1968). Rather, a yellow xanthophore lies above an iridophore, and the two are above cup-shaped melanophores. Some of the incoming light is scattered into the blue wavelength by the iridophore, and the remaining light is absorbed by the melanophores. The blue light that is reflected back must first pass through a layer of yellow xanthophores, which makes the frog appear green. Bagnara, Taylor, & Hadley (1968) produced blue colored skin by leaching out the xanthophore pigments with alcohol.

Berns and Narayan (1970) used solvent extractions and absorption spectrophotometry to compare blue and green areas of skin on variant blue frogs of *Rana clamitans*. They found carotenoids were greatly reduced, and xanthophores were either absent, or present but lacking carotenoid vesicles in blue areas of the skin. Nishioka and Ueda (1985) report a remarkable occurrence in *Rhacophorus schlegelii*. Some albinos of this species are green. Green color can develop because certain xanthophores sink down into spaces below iridophores and turn into violet-black violophores. This allows the iridophores to reflect blue and the frog to appear green, despite the absence of melanin. Lyerla and Jameson (1968) found no noticeable difference in the arrangement cell types in brown and gray skin, but xanthophores were much less abundant in gray than in brown skin.

Although all frogs can lighten or darken existing skin tones, actual color change (e.g. from uniform green to brown) is uncommon. During the culture of xanthophores and iridophores from *Rana catesbeiana*, Ide (1974; 1978) recognized the appearance of melanophores and thus demonstrated *in vitro* plasticity of pigment cells. The extent to which this change happens *in vivo* is unknown. Color change observed in nature is usually age-related and in one direction. The exceptions are *Hyla regilla* and *Acris crepitans*. In *H. regilla,* individuals have been observed to change from brown to green, or vice versa, over periods of weeks to months under laboratory conditions (pers. obs.; Mackey, 1958; Haugen, 1992). Color change is reversible in some individuals under some environmental conditions (W. Wente, pers. comm.). In *A. crepitans*, individuals have been observed to change from green stripe to gray stripe or vice versa over a six month period (Pyburn, 1961b).

Age-related (ontogenetic), directional changes in CP have been observed in at least 39 species (Table 2.3), over half of which belong to the genus *Hyperolius*. Examples include *Amphignathodon guentheri*, which are uniformly green as juveniles and tan or dark brown-gray as adults (Duellman & Ruiz-C, 1986), and *Gastrotheca aureomaculata*, which are uniformly green as juveniles, and become brown/olive and spotted as adults (Duellman, 1983; Duellman & Ruiz-C, 1986). The most studied (and perhaps most interesting) anurans that undergo ontogenetic

change are species in the genus *Hyperolius*. Several species in this genus undergo a characteristic ontogenetic change from a juvenile morph into an adult. Sometimes only adult females change color [e.g. *Hyperolius v. viridiflavus* metamorphs have a tan dorsum with darker tan stripes, while all females and some males develop into bright green frogs with yellow spots (Richards, 1976)]. Richards (1982) treated *H. v. viridiflavus* tadpoles with daily estrogen or testosterone baths and found that all treated tadpoles metamorphosed with the adult green phenotype. Hayes and Menendez (1999) compared development in, and affects of exogenous steroid hormones on two species, *H. v. viridiflavus* (which is sexually monomorphic) and *H. argus* (which is sexually dimorphic). Although their results for *H. v. viridiflavus* were identical to those of Richards (1982), they found that in *H. argus*, only estrogen treatments induced adult coloration in metamorphs. Testosterone treatments had no effect. Hayes and Menendez (1999) conclude that sexual dichromatism may evolve by two mechanisms, depending on the state of the ancestral condition. If monomorphism is ancestral, then dichromatism may evolve with a loss of responsiveness to testosterone, so that males no longer change color. If dichromatism is ancestral, then ontogenetic change in both sexes may result from the acquisition of the ability to respond to androgens as well as estrogens. Finally, many anurans tend to darken with age and/or size (e.g. *P. mascareniensis*, *R. pipiens*, and *R. clamitans* - Richards and Nace, 1983; *R. septentrionalis* - Kramek & Stewart, 1980), although this is not color change *per se*. In the case of *R.*

*septentrionalis*, age-related darkening is caused by a relative increase in spot size rather than by an increase in spot number (Kramek & Stewart, 1980).

The data on color change suggest the following research priorities. First, although it is likely that hormonal changes control ontogenetic change in many species, few details have been worked out (Richards, 1982; Hayes & Menendez, 1999). The endocrinology of anuran color development and/or change appears to be a wide-open area of research. Second, changes between green and brown states seem to be a particularly common transition (*A. guentheri*, *G. aureomaculata*, *G. griswoldi*, *Hyperolius viridiflavus*, *Hyla regilla*). More work on the proximate environmental and physiological control of green-brown polymorphisms would shed light on the opportunity for adaptive plasticities, and on potential limits to the heritability of these polymorphisms. Finally, there has been no work on whether ontogenetic color change is adaptive in any anuran species.

## SEXUAL DIMORPHISM

There are twenty-five examples of anurans that are sexually dimorphic for CP (Table2. 4). Not surprising, the literature does little more than describe the nature of the polymorphism for the majority of these species. *Bufo periglenes* provides a dramatic example of sexual dimorphism in anurans, with bright orange males and females that are a cryptic olive/black with red spots (Savage, 1966). Unfortunately, this species is probably now extinct (Pounds & Crump, 1994).

Table 2.3: Species for which there is a record of ontogenetic change in color or pattern.

| Species | Description | Reference |
|---|---|---|
| *Acris crepitans* | Gray stripe in metamorphs may change to red or green. | Gray, 1972 |
| *Agalychnis callidryas* | Spotted coloration does not develop until several weeks after metamorphosis. | Starret, 1960; Duellman 1983 |
| *Amphignathodon guentheri* | Juveniles are always uniform green, adults are tan or dark brown-gray. | Duellman & Ruiz-C, 1986 |
| *Bufo houstonensis* | Juveniles are beige, adults are gray/white | Mays & Reed, 1985 |
| *Dendrobates leucomelas* | Born with yellow bands around waist, black spots develop within yellow band as they mature. | Mattison, 1998 |
| *Eleutherodactylus polychrus* | Juveniles have orange-red colors on the hidden parts of the legs and a yellow venter. Adult females have black-gray on the hidden parts of the legs and a | Ruiz et al., 1996 |

| | | |
|---|---|---|
| | black-gray venter. | |
| *Gastrotheca aureomaculata* | Juveniles are always uniform green, adults are brown or olive to green. Additionally, spotted coloration does not develop until several weeks after metamorphosis. | Duellman, 1983; Duellman & Ruiz-C, 1986 |
| *Gastrotheca griswoldi* | Young juveniles are uniform green, old juveniles are uniform brown to gray. | Duellman & Ruiz-C, 1986 |
| *Gastrotheca helenae* | Juveniles are always light tan, adults are tan to dark brown. | Duellman & Ruiz-C, 1986 |
| *Hyla regilla* | Some individuals have the ability to change completely from brown to green, or vice versa. | Mackey, 1958; Pers. obs. |
| *Hylactophryne augusti* | Born with white bands around body, these bands fade over time. | Mattison, 1998 |
| *Hyperolius sp.* | A general pattern of ontogenetic pattern is present in many *Hyperolius* species (see table 1 for descriptions). | See table 1 |
| *Leptopelis concolor* | Juveniles are green, females are | Passmore & |

| | | |
|---|---|---|
| | tan to dark brown | Carruthers, 1979 |
| *Pseudacris ornata* | After metamorphosis many juveniles were copper in color, later on they developed into other colors. | Harkey & Semlitsch, 1988 |
| *Ptychadena mascrareniensis* | Increase in pigmentation over time may lead to dark variants. | Richards & Nace, 1983 |
| *Rana clamitans* | Increase in pigmentation over time may lead to dark variants. | Richards & Nace, 1983 |
| *Rana pipiens* | Increase in pigmentation over time may lead to dark variants. | Richards & Nace, 1983 |
| *Rana septentrionalis* | Always an increase of dark pigmentation over time. | Kramek & Stewart, 1980 |

The most well studied taxon having sexually dimorphic species is the genus *Hyperolius*. Examples are described above.

Although few have speculated on the advantages of sexual dimorphism in anurans, one interesting hypothesis for *Hyla luteocellata* (Rivero, 1969) is that when a pair is in amplexus the different striping patterns meld to form the illusion of one large frog. A more general hypothesis is that sexual niche-partitioning exists (Shine, 1989). Hayes (1997) suggested that sexual dimorphism may exist in *Hyperolius argus* because males are at greater risk in breeding ponds where they vocalize to attract females, therefore green color may provide better camouflage at the breeding sites. One interesting question is why sexual dimorphism in color or pattern appears to be less common in anurans than in other vertebrates, such as birds. The most obvious explanation is that most anurans breed at night, which reduces the opportunity for sexual selection on CP traits. Interestingly, *B. periglenes* and *B. canorus* may breed during the day. Nevertheless, there have been no studies comparing the ecology or behavior of dimorphic and non-dimorphic species.

## MODES OF INHERITANCE

The inheritance of CP polymorphisms has been investigated in twenty-six species (Table 2.5), but conclusive data on the mode of inheritance are available for only a handful of these. Although there are numerous species in which extremely

rare variants have been found or created by irradiation, this section is limited to true polymorphisms as defined in the Introduction. Studies on the inheritance of anuran CP polymorphisms fall into three main categories, which I can list in order of increasing strength of inference:

(1) Most studies have examined morph ratios in offspring from single generation clutches that were either field-collected (Lynn & Lutz, 1947; Ishchenko & Shchupak, 1974; Church, 1963) or produced via deliberate crosses between wild-caught animals (Pyburn, 1961a; Resnick & Jameson, 1963; Main, 1965; Bruce & Parkes, 1947; Travis & Trexler, 1984; Mathews & Pettus, 1966; Goin, 1947; Moriya, 1952; Moore, 1942; Volpe, 1955; Browder, Underhill, & Merrell, 1966; Browder, 1968; Corn, 1986). Such data are useful for generating hypotheses about inheritance. However, many of these studies went on to use the same data to test those hypotheses, which is an obvious violation of statistical inference. Even more surprising is how uncritically subsequent authors have accepted the conclusions of many of these studies. For example, Lyerla and Jameson (1968) stated that for *Hyla regilla*, "Green has been shown to be the result of a pair of dominant genes (O_G_) in enzyme sequence, while non-green results if either locus is homozygous recessive (oo or gg)." This statement has been repeated in the literature for years. However, if one examines the original data (Resnick & Jameson, 1963), one can

Table 2.4: Species for which there is a record of sexual dimorphism in color or pattern polymorphism.

| Species | Description | Reference |
|---|---|---|
| *Boophis microtympanum* | Males are green, females are yellow-green. | Glaw & Vences, 1992 |
| *Boophis pauliani* | Males are green, females are brown. | Glaw & Vences, 1992 |
| *Bufo canorus* | Male: uniform dorsal yellow-green to olive. Female: gray, tan, or brown, with black spots. | Karlstrom, 1973 |
| *Bufo periglenes* | Male: uniform bright orange. Female: olive to black with red spots. | Savage, 1966 |
| *Bufo taitanus* | Males: middorsal pattern of 3 dark blotches. Females: light reddish-brown dorsum with dark flanks. | Stewart, 1967 |
| *Eleutherodactylus bicolor* | Males: red color on hidden parts of legs. Females: yellow color on hidden parts of legs. | Rueda & Lynch, 1983 |
| *Eleutherodactylus erythropleura* | Males: hidden area of legs with yellow spots. Females: hidden area | Lynch, 1992 |

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| | of legs with orange-pink spots. | |
| *Eleutherodactylus factiosus* | Males: hidden area of legs with yellow spots. Females: hidden area of legs with orange-pink spots. | Lynch & Rueda, 1998a |
| *Gastrotheca andaquiensis* | Males are brown, females are green | JD Lynch, pers. comm. |
| *Heterixalus variabilis* | Males are without conspicuous coloration, females are beige to yellow with large black markings | Glaw & Vences, 1992 |
| *Hyla bokermanni* | Male: lateral markings. Female: dorsolateral striped. | Rivero, 1969 |
| *Hyla luteocellata* | Male: lateral markings. Female: dorsolateral striped. | Rivero, 1969 |
| *Hyperolius argus, H. castanus, H. cinnamomeoventris, H. discodactylus, H. glandicolor, H. karissimbiensis, H. kivuensis, H. mariae, H. marmoratus, H. nasutus, H. ocellatus,* | For descriptions see Table 1. | See Table 1 |

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| *H. pleurosphilus, H. quinquevittatus, H. spatzi, H. tuberculatus, H. tuberilinguis, H. v. viridiflavus* | | |
| *Leptopelis concolor* | Juveniles are green, females are tan to dark brown. Males occur as both phenotypes. | Passmore & Carruthers, 1979 |
| *Rana septentrionalis* | Females have dark pigmentation in a reticulate pattern that males do not have. | Kramek & Stewart, 1980 |
| *Taychynemis seychellensis* | In some populations males are brown, females are green. | Nussbaum & Wu 1995 |

Table 2.5: Species for which the inheritance and/or genetic mechanisms of a color polymorphism have been investigated. See text for explanation of categories of inference 1, 2, and 3; O = Other types of inference used to determine genetic basis of polymorphism.

| Species | Category of Inference | Hypothesized mode of inheritance (where applicable) | Reference |
|---|---|---|---|
| *Acris crepitans* | 1 | Green stripe is dominant to lack of stripe | Pyburn, 1961a |
| *Acris crepitans* | 2 | Red is permanent; green may change to gray; green is dominant to gray; red is dominant to gray | Pyburn, 1961b |
| *Bombina orientalis* | 3 | Pale is recessive to wild type | Ellinger, 1980 |
| *Crinia georgiana, glauerti, insignifera, pseudoinsignifera* | 1 | <u>Same for all 4 species:</u><br>One locus, 2 alleles;<br>Ridged = Homozygous<br>Lyrate = Heterozygous<br>Weak Lyrate = Homozygous | Main, 1965 |

| | | | |
|---|---|---|---|
| *Crinia (Ranidella) glauerti, insignifera, pseudoinsignifera* | 2 | Same for all 3 species<br>One locus, 2 alleles;<br>Lyrate = dominant<br>Ridged = recessive | Bull 1975 |
| *Discoglossus pictus* | 1 | One locus, striped allele dominant to spotted allele | Bruce & Parkes, 1947 |
| | 3 | One locus, striped allele dominant to spotted allele | Lantz, 1947 |
| *Eleutherodactylus alticola* | 2 | Inheritance of middorsal stripe is unclear; dorsolateral stripe presence is dominant to absence | Goin, 1950 |
| *Eleutherodactylus bransfordii* | O | One locus, ridging is caused by 3 alleles (parallel and U-shaped are recessive, and scattered is dominant) | Savage & Emerson, 1970 |
| *Eleutherodactylus nasutus* | 2 | One locus, dorsolateral stripe is dominant to non-stripe | Goin, 1950 |
| *Eleutherodactylus nubicola* | O | Dorsolateral stripe and middorsal stripe are dominant to absence | Goin, 1960 |
| *Eleutherodactylus pantoni* | 2 | Dorsolateral stripe and middorsal stripe are dominant to absence | Goin, 1950 |

| | | | |
|---|---|---|---|
| *Hyla regilla* | 2 | Red allele recessive to brown at one locus; green color may be controlled by dominant alleles at two other loci. One can rule out a single locus with green dominant | Resnick & Jameson, 1963 |
| *Pseudacris ornata* | 1 | Genetic basis to color, but also environmental effects on determination of brown vs. gray | Travis & Trexler, 1984 |
| | O | Alteration of temperature in the larval environment may explain some variation in brown vs. gray morph frequency | Harkey & Semlitsch, 1988 |
| | 2 | Green is controlled by single dominant allele over brown/gray | Blouin, 1989b |
| *Pseudacris triseriata* | 1 | 3 loci, with all red and green dominant to recessive brown | Mathews & Pettus, 1966 |
| | 1 | One locus, albinism recessive | Corn, 1986 |
| *Ptychadena mascareniensis* | O | Dark variant is caused by dominant gene | Richards & Nace, 1983 |
| *Rana arvalis* | 1 | One locus, stripe (striata) is dominant to spotted (maculata) | Ishchenko & Shchupak 1974 |

| | | | |
|---|---|---|---|
| *Rana clamitans* | O | Dark variant is caused by dominant gene | Richards & Nace, 1983 |
| *Rana limnocharis* | 2 | One locus, with allele for dorso-median stripe dominant to non-striped | Moriwaki, 1953 |
| *Rana nigromaculata* | 1 | One locus, white ventral skin is dominant to dark markings on ventral skin | Moriya 1952 |
| *Rana pipiens* | 1 | One locus, burnsi dominant to wild-type | Moore, 1942 |
| | 2 | One locus, burnsi dominant to wild-type | Moore, 1943 |
| | 1 | One locus. kandiyohi dominant to wild-type | Volpe, 1955 |
| | 2 | Kandiyohi and burnsi are simple dominant to normal spot pattern but lack dominance with respect to each other. Double dominant gives mottled burnsi. | Volpe, 1960 |
| | 1 | One locus, kandiyohi mutation is incompletely dominant with variable expression | Browder, 1968 |

| | | | |
|---|---|---|---|
| | 2 | One locus, green dominant to brown | Fogleman et al., 1980 |
| | O | Dark variant is caused by dominant gene | Richards & Nace, 1983 |
| *Rana sylvatica* | 1 | One locus, striped dominant to unstriped | Browder et al., 1966 |
| | O | Dark variant is caused by dominant gene, but penetrance is low so in many cases looks recessive | Richards & Nace, 1983 |
| *Rhacophorus leucomystax* | 1 | Inheritance is controlled by multiple genes | Church, 1963 |

only conclude that color is heritable (i.e. green parents tend to give green offspring, brown tend to give brown) and that one can rule out a single locus with green dominant (e.g. brown x brown crosses sometimes give green offspring).

(2) Much stronger inference is achieved in studies that used one set of crosses to generate hypotheses, and a second, *independent* set of wild crosses to test those hypotheses (Moriwaki, 1953; Fogleman, Corn, & Pettus, 1980; Blouin 1989b; in some cases the tested hypotheses were posed by other authors: Pyburn, 1961b; Bull, 1975; Goin, 1950; Moore, 1943). Nevertheless, these studies all involve a single generation of offspring.

(3) Multigeneration breeding studies are necessary to prove a mode of inheritance absolutely because complicated modes of inheritance, such as quantitative threshold traits (Wright, 1934), can mimic simple Mendelian ratios in the F1 generation. Mode of inheritance has been demonstrated via multigeneration breeding studies in only two species, *Discoglossus pictus* and *Rana pipiens*. Lantz (1947) conducted eight crosses between various combinations of striped and spotted *D. pictus*, and then conducted five F1 backcrosses to prove that the striped pattern is controlled by a dominant allele at a single locus. Many investigators have studied the inheritance of spotting patterns in the common laboratory frog, *R. pipiens* (Table 2.5). Initially, the *R. pipiens* complex was thought to consist of three distinct species based on back pattern: *R. pipiens* (spotted), *R. burnsi* (unspotted),

and *R. kandiyohi* (mottled; Weed, 1922). Eventually it was demonstrated that dominant alleles at each of two unlinked loci control the rare burnsi and kandiyohi phenotypes in a single species (e.g. Volpe, 1956; Volpe, 1961a; Anderson & Volpe, 1958). Further work suggested that modifier loci and environmental effects influence the expression of the burnsi allele (Volpe, 1961a; Davison, 1964; Browder & Davison, 1964). There also appears to be an interaction between the dominant alleles at the two loci because there is a deficiency in kandiyohi progeny when eggs produced by burnsi females are fertilized by sperm from kandiyohi males (Nace, Richards, & Asher, 1970; Merrell, 1972). Finally, a recessive allele at a third locus alters the differentiation of iridophores and xanthophores so that homozygous frogs are melanistic dorsally and transparent ventrally (Richards, Tartof, & Nace, 1969). However, this morph is rarely found in nature.

Despite the lack of conclusive data on inheritance in most species, most of the above studies at least suggest that CP variation is highly heritable. If one takes the conclusions of the above studies at face value, then some interesting patterns emerge. First, in species that exhibit a rare morph, the allele causing the rare morph is usually dominant (*A. crepitans*, *Eleutherodactylus* sp., *Pseudacris ornata*, *P. triseriata*, *Rana pipiens*, and *R. sylvatica*) (also noted in Duellman & Trueb, 1986). Second, the presence of the middorsal stripe is usually caused by a dominant gene (*A. crepitans*, *D. pictus*, *P. ornata*, *Eleutherodactylus nasutus*, *E. nubicola*, *E. pantoni*, *Rana arvalis*, *R. limnocharis*, and *R. sylvatica*). Third, in four of the five species where green morph inheritance has been investigated, the green color

appears dominant to the gray/brown color (*A. crepitans*, *H. regilla*, *P. ornata*, *R. pipiens*, but not *P. triseriata*). Fourth, light colored or albinistic morphs are caused by a recessive allele in six of the seven species investigated, as is typical for other vertebrates (*B. orientalis*, *Ptychadena mascareniensis*, *R. clamitans*, *R. pipiens*, *R. sylvatica*, *P. triseriata*, but not *R. nigromaculata*). Finally, a gross environmental effect has only been demonstrated in two species (spot number in *R. pipiens*: Browder & Davison, 1964; Davison, 1964; Cassler, 1967; brown/gray ratio in *P. ornata*: Travis & Trexler, 1984; Harkey & Semlitsch, 1988), although few studies have tested for it. Thus, data on modes of inheritance of CP polymorphisms in more species of anurans are needed. This step is necessary before meaningful studies on selective maintenance of these polymorphisms can be conducted.

## SELECTIVE MAINTENANCE

### *Evidence for selection*

There have been no direct demonstrations of selection on an anuran CP polymorphism in the wild using marked individuals followed over time. That polymorphisms are under selection has been inferred from three types of observations:

(1) Seasonal fluctuations in morph frequencies have been invoked as evidence for selection. For example, Mathews (1971) found that, for three years in a row, the percentage of green-spotted morph in breeding adult *P. triseriata* was higher than in their offspring at metamorphosis. Similarly, Jameson and Pequegnat (1971) found that the percentage of green *H. regilla* increased over the breeding season in two of three years. A similar class of studies infers selection from the observation that age classes in a static sample differ in morph frequency (Ishchenko, 1994). Of course, ontogenetic color change (Table 2.3) or change in response to environment (known to occur for green/brown polymorphisms in *H. regilla* and *A. crepitans*), could seriously affect these sorts of studies. One must also consider the effective number of breeders, Nb, each generation, which can be substantially smaller than the number of males and females (e.g. Scribner, Antzen, & Burke, 1997). Small Nb could cause large variation in allele frequencies between age classes.

(2) Examples of non-random survival of morphs during episodes of high mortality provide much more compelling evidence of selection. For example, when disease swept through three tanks holding *A. crepitans*, the gray morph survived best in all three tanks (Nevo, 1973). Here sample sizes were large and each tank held animals from a different population, so any confounding variables are hard to pinpoint. Similarly, Merrell and Rodell (1968) observed a higher proportion of burnsi *Rana pipiens* among living than dead frogs in the field after an episode of winter mortality. That % burnsi was higher in a spring sample of frogs than in a sample

from the previous autumn is also consistent with higher burnsi overwintering survival. In a separate incident, Dapkus (1976) was overwintering 1,598 *R. pipiens* in outdoor containers when a bout of warm weather killed one third of them. Again, burnsi morphs were over represented in the survivors. As compelling as these data appear, however, they are not the result of planned experiments. It is surprising that no one has tested these observations with planned experiments using marked individuals and insuring that no other variables, such as family, are confounded with morph type. Nevertheless, that anuran CP polymorphisms are under some sort of balancing selection seems likely. There are so many polymorphic species that it is hard to believe each is simply in a transition between fixation of alternate neutral alleles.

(3) Another way selection has been inferred is from apparent convergent evolution between distantly related species (Volpe, 1957; Milstead, Rand, & Stewart, 1974; Richards & Nace, 1983; Blouin, 1989a). Here the argument is that similar selection pressures have caused the evolution of similar but non-homologous polymorphisms in unrelated species. For example, the dorsal pattern polymorphisms of *D. pictus*, *R. limnocharis*, and *R. nigromaculata* are very similar, as are the spotting patterns of *R. pipiens* and *E. ricordii* (Volpe, 1957). Milstead et al. (1974) suggested that five genera of anurans, each on a different continent, fill the same niche and exhibit similar polymorphisms, thus supposedly showing the convergence of a selectively beneficial trait. A green/brown/gray polymorphism also appears to be a common

cross-taxon phenomenon. At least twenty-two species from 14 different genera exhibit this striking color polymorphism (including: *Acris crepitans, A. gryllus, Arthroleptis adolfifriederici, Cacosternum boettgeri, Hyla regilla, Hyperolius argus, H. v. viridiflavus, Leptopelis flavomaculatus, L. vermiculatus, Limnonectes tigerinus, Mantidactylus peraccae, Pseudacris ornata, P. triseriata, Rana pipiens, R. septentrionalis, Rhacophorus leucomystax*). The existence of this similar polymorphism in such a wide array of species suggests that both homology and convergence may influence this phenotypic character. The occurrence of identical polymorphisms in closely related species raises the intriguing possibility that ancestral polymorphisms have been maintained by selection for thousands or millions of years (Bull, 1975). For example, Main (1965) and Bull (1975) demonstrated, via interspecific crosses, that pattern polymorphism in different species of *Crinia* is controlled by homologous loci. Homology has not been tested in other cases of identical polymorphisms (e.g. Milstead et al., 1974; Cei, 1959; Volpe, 1957; Goin, 1954; Lynch, 1966; Goin, 1950; Stewart, 1974; Blouin, 1989a, Brown & Funk, 1977; Richards & Nace, 1983) thus convergent evolution cannot be ruled out.

*Mechanisms of selection*

The most plausible mechanisms for maintaining a balanced polymorphism in nature are heterozygote advantage, frequency dependent selection, and spatial

variation in selection pressure (Hartl & Clark, 1997; Hedrick, Ginevan, & Ewing, 1976; Hedrick, 1986). Discussions of the maintenance of anuran polymorphisms invoke either selection directly on CP itself, or selection on traits genetically correlated with CP. These studies generally take one of the following approaches (Table 2.6):

(1) A common approach has been to observe a correlation between spatial or temporal variation in some environmental variable and variation in morph frequencies. For example, variation in morph frequencies among populations has been found to correlate with the color and/or pattern of substrate and vegetation at pond edges (*Acris* sp.-Nevo, 1973; *Eleutherodactylus bransfordii*-Savage and Emerson, 1970; *Hyla regilla*-Test, 1898; Haugen, 1992; *Phrynobatrachus* sp.-Stewart, 1974; *Pseudacris triseriata*-Tordoff, Pettus, & Mathews, 1976; Tordoff & Pettus, 1977; *R. pipiens*-Bresler, 1963; *R. septentrionalis*-Kramek & Stewart, 1980; *R. sylvatica*-Fishbeck & Underhill, 1971; Schueler & Cook,

Table 2.6: Species for which selective mechanisms maintaining a polymorphism were investigated. See text for description of codes of the approaches used to detect selection.

| Species | Approach used to detect selection | Data and/or Hypothesized Results | Reference |
|---|---|---|---|
| *Acris crepitans* | 1 | Red-stripe and green-stripe morphs favored in wet season, gray morph favored during dry season | Pyburn, 1961b |
| | 3 | No differential predation by birds on different color morphs; lab test | Wendelken, 1968 |
| | 1 | Difference in phenotypic frequencies was stable over a 12 week time period (summer to fall) | Isaacs, 1971 |
| | 1,2 | Correlation between morph and substrate color | Nevo, 1973 |
| | 2 | Measured various physiological factors and found no differences between morphs | Gray, 1977 |

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| | 3 | No difference of predation on, and substrate preference of red, green, gray, stripped morphs | Gray, 1978 |
| | 2 | Annual and geographic differences in frequency, but no seasonal differences. | Gray, 1983 |
| *Acris gryllus* | 1,2 | Correlation between morph color and ecological variables. Differences between morphs in disease resistance | Nevo, 1973 |
| *Crinia georgiana, glauerti, insignifera, pseudoinsignifera* | 1,2 | Same for all 4 species: All populations show a decline in frequency of the lyrate morph between March and November. Conclude: Cold season – lyrate favored Warm season – ridged favored | Main, 1968 |
| *Crinia (Ranidella) insignifera* | 2 | During maturation to adulthood, higher proportions of lyrate morphs survived cool summers | Main, 1961 |
| *Crinia (Ranidella) insignifera* | 2 | Measured heat tolerance and water loss and concluded that ridged | Walker, 1966 |

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|---|---|---|---|
| | | animals have an advantage under warm conditions | |
| *Crinia (Ranidella) insignifera, pseudoinsignifera* | 2 | Same for both species: Ridged morphs metamorphose faster than lyrate morphs | Bull, 1977 |
| *Eleutherodactylus bransfordii* | 1 | Morph frequency is correlated with latitude. Different morph frequencies are correlated with amount of light on forest floor | Savage & Emerson, 1970 |
| *Hyla regilla* | 2 | Predictable variation in color frequencies within a year; also suggest difference in fecundity between morphs | Jameson & Pequegnat, 1971 |
| | 1 | Frequency of green morphs highest in the spring | Schaub & Larson, 1978 |
| | 3 | Higher predation on morphs that did not match substrate in lab | Morey, 1990 |
| *Phrynobatrachus acridoides, natalensis, ukingensis* | 1 | Same for all 3 species: Geographic variation in morph frequency | Stewart, 1974 |

| | | | |
|---|---|---|---|
| *Pseudacris ornata* | 2 | No difference between color morphs in larval period, size at metamorphosis, size at day 46, or adult size | Travis & Trexler, 1984 |
| | 2 | No difference between color morphs in larval or juvenile fitness correlates | Blouin, 1989a |
| *Pseudacris triseriata* | 2 | Suggest individuals with brown spot have better survival in aquatic phase; green spot have better survival in terrestrial stage. Green metamorphose earlier | Mathews, 1971 |
| | 1 | Spatial variation in morph frequency across 7 ponds | Tordoff et al., 1976 |
| | 1 | No significant fluctuations in dorsal color frequencies occurred over five years; lab study | Tordoff & Pettus, 1977 |
| | 3 | Jays selectively preyed upon frogs that did not match substrate | Tordoff, 1980 |

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|---|---|---|---|
| | 2 | Measured many fitness correlates and concluded that differences in rate of development existed between color morphs in later juvenile stages. | Hoppe & Pettus, 1984 |
| *Rana arvalis* | 2 | Larval growth rates are same for both morphs, but maculata adults breed earlier and therefore their offspring emerge first. | Ishchenko & Shchupak, 1974 |
| *Rana pipiens* | 1 | Observed a large geographic range distribution of the burnsi morph. | Bresler, 1963 |
| | 2 | Suggests that burnsi adults are better able to survive winter than normal | Merrell & Rodell, 1968 |
| | 2 | Found that B/+ heterozygotes may have advantage over +/+ in survival | Gill, 1970 |
| | 2 | Kandiyohi larvae have more rapid development than wild type | Merrell, 1972 |
| | 2 | Observed differential morph survival when captive frogs died due to unusually warm temperature. | Dapkus, 1976 |

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| | 2 | Rough estimate of larval period suggested that brown morphs matured quicker | Corn, 1981 |
| *Rana septentrionalis* | 1 | Observed correlation between color and habitat choice | Kramek & Stewart, 1980 |
| *Rana sylvatica* | 1 | Observed a southeast to northwest gradient in dorsal stripe, with lowest frequencies in southeast | Fishbeck & Underhill, 1971 |

1980). Similarly, short term changes in morph frequencies within a site have been hypothesized to be caused by changing vegetation cover (*A. crepitans*-Pyburn, 1961b; Isaacs, 1971; *Crinia* sp.-Main, 1968; *H. regilla*-Schaub & Larson, 1978; *P. triseriata*-Mathews, 1971; *Rana arvalis*-Ishchenko, 1994; *R. pipiens*-Merrell, 1965). Here the inferred mechanism is fluctuating selection by visual predators directly on CP (caused by changing substrate color). For example, Fishbeck and Underhill (1971) found a clinal change in dorsal stripe frequency in *R. sylvatica.* They hypothesized that the striped phenotype has a selective advantage in coniferous forested areas and ponds with emergent vegetation because both of these variables correlated with the westerly increase in stripe frequency.

(2) A second approach has been to simply look for fitness-related traits that are correlated with CP polymorphisms. Various correlates of CP have been proposed or tested, including susceptibility to desiccation, stress resistance, emergence of adults, disease, fecundity, mating preference, length of larval period, size at metamorphosis, juvenile size, adult size, tadpole growth rate, and survival during different seasons (*Acris* sp.-Nevo, 1973; Gray, 1977; Gray, 1983; *Crinia* sp.-Main, 1961; Main, 1968; Walker, 1966; Bull, 1977; *H. regilla*-Jameson & Pequegnat, 1971; *P. ornata*-Travis & Trexler, 1984; Blouin 1989a; *P. triseriata*-Mathews, 1971; Hoppe & Pettus, 1984; *Rana arvalis*-Ishchenko & Shchupak, 1974; *R. pipiens*-Merrell, 1965; Merrell & Rodell, 1967; Gill, 1970; Merrell, 1972; Dapkus, 1976; Corn, 1981). Most of these studies found no correlation between morph and

trait, were anecdotal in nature, or were so flawed in experimental design that the results are difficult to evaluate. Additionally, one study (Hoppe & Pettus, 1984) measured 9 variables and found two statistically significant correlations, but these disappear when a correction for multiple comparisons is made. Nevertheless, a few compelling examples of fitness traits correlated with CP morph exist. The survival difference between *R. pipiens* morphs suggests a correlation with some tolerance for physiological stress (Merrell and Rodell, 1968; Dapkus, 1976). Similarly, Nevo's (1973) data suggest a correlation with disease resistance in *A. crepitans*. There appears to be a correlation between decreased length of larval period and the ridged morph in *Crinia* sp. (Bull, 1977; Walker, 1966; Main, 1965). Although none of these three studies on *Crinia* provide conclusive evidence for a genetic correlation on their own, it is suggestive that all three independently found the same result. Finally, Merrell (1972) conducted three independent crosses of kandiyohi heterozygotes with wild type *R. pipiens* and found that kandiyohi heterozygotes always had a decreased larval period.

(3) Only four studies used an experimental approach to test for direct selection on the polymorphism itself. Wendelken (1968) allowed adult great-tailed grackles, *Cassidix mexicanus*, to prey upon green, gray, and red morphs of *A. crepitans* on a background of grass and soil. Gray (1978) tested predation susceptibility of *A. crepitans* in a 20 gallon aquarium with different substrates. He used juvenile bullfrogs (*R. catesbeiana*), garter snakes (*Thamnophis sirtalis* and *T. radix*), and

northern water snakes (*Nerodia sipedon*) as predators. Tordoff (1980) tested whether or not gray jays, *Perisoreus canadensis*, would prey differentially on brown or green morphs of *P. triseriata* depending on background color (dyed terrycloth). Finally, Morey (1990) used a setup similar to Tordoff's (1980) to test whether garter snakes (*T. elegans*) preferentially attack *H. regilla* morphs that do not match the substrate color. Of these four studies, two found that predators preferentially ate non-matching morphs (Tordoff, 1980; Morey, 1990). All four of these experiments were conducted in a laboratory setting.

So what general conclusions can one draw about the form of natural selection maintaining these polymorphisms? First, there have been no direct tests of heterozygote advantage. In most species for which the inheritance of the polymorphism is understood, heterozygotes are indistinguishable from homozygous dominants, so any heterozygote advantage would obviously have to be expressed in a correlated trait. Second, one of the most frequently invoked mechanisms to maintain anuran polymorphism is temporal variation in selection pressures (e.g. morph "A" favored as tadpole, morph "B" favored as adult). Unfortunately, the conditions necessary for a polymorphism to be maintained in such a manner would rarely be met (Hedrick et al., 1976, Hedrick 1986). Third, selection on correlated traits is plausible, but a large number of studies and traits have turned up little evidence. Also, these "correlated trait" explanations are very ad hoc. At least two other problems emerge from the correlational data. First, correlational data do not show causation, which is sometimes assumed. Second, the

presence of unrecognized species may account for the correlation between phenotypic variation and an environmental variable such as habitat locality or time of emergence (J. Lynch, pers. comm., e.g. Mathews, 1971; Savage & Emerson, 1970; Bresler, 1963; Cochran & Goin, 1970). There are so many species of anurans sporting striking CP polymorphisms, that it seems unlikely there is a common correlate of CP, such as desiccation tolerance or larval development rate. It seems even less plausible that a different correlated trait maintains the polymorphism in each species. Thus, direct selection on CP itself by visually oriented predators still seems the most likely explanation given anurans' position at the bottom of the vertebrate food chain. For species with apostatic predators (predators that prey differentially upon more common phenotypes), polymorphism reduces the overall predation risk for any one individual (Allen, 1988). For example, an apostatic predator may switch to an entirely different monomorphic species because the alternate prey appears more common (Endler, 1991). Nevertheless, there have been few experimental tests of the selective predation hypothesis, and none were conducted in the field. Most of the data on selection on anuran CP polymorphisms remains correlative or anecdotal, and even for the most well-studied species there is little consensus on how the polymorphisms are maintained.

## SUMMARY

There are at least 225 species representing 35 genera and 11 families of anurans that exhibit a color or pattern polymorphism. These include several apparent cases of parallel polymorphism or convergent evolution. This diversity provides an excellent system in which to study polymorphism.

The mode of inheritance has been investigated in 26 species. Although most studies indicate that CP is highly heritable, simple Mendelian inheritance has been conclusively demonstrated for CP polymorphisms in only 2 species (not counting albinos and other variants that are too rare to qualify as polymorphisms). There is an obvious need for more genetic studies.

Forty-six species have been described as undergoing ontogenetic change, and thirty-two species have been described as sexually dimorphic. An interesting area of research would be on the adaptive benefits of ontogenetic change, and on why anurans are sexually dimorphic. That anuran CP polymorphisms are under some sort of selection has been inferred by the large number of polymorphic species, the occurrence of apparent convergent evolution and the existance of identical polymorphisms in closely related species, the observation of consistent cyclical fluctuations in frequencies of morphs over short (seasonal) or long (annual) periods of time, and a few fortuitous cases of non-random survival (*A. crepitans*, and *R. pipiens*).

The mechanisms behind the selective maintenance of color and/or pattern polymorphism have been investigated in 19 species. Most of these studies attempted to determine if some fitness-related trait was correlated with morph type, and were mostly inconclusive. Although only four studies investigated direct selection by predators on CP polymorphism itself, two of these demonstrated differential selection (albeit in the laboratory). I believe that the simplest explanation for the maintenance of CP polymorphisms is indeed direct selection by visually-oriented predators. Well-designed studies are needed on the effects of predation on morph frequencies in the field. Although such studies are difficult to conduct, they are likely to be much more fruitful than yet more searching for correlated physiological traits. This remains a wide open and potentially rewarding area of study.

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# CHAPTER 3.
# EVOLUTIONARY HISTORY OF THE NORTHERN LEOPARD FROG: RECONSTRUCTION OF PHYLOGENY, PHYLOGEOGRAPHY, AND HISTORICAL CHANGES IN POPULATION DEMOGRAPHY FROM MITOCHONDRIAL DNA

ABSTRACT

This study uses a combined methodological approach including phylogenetic, phylogeographic, and demographic analyses to understand the evolutionary history of the northern leopard frog, *Rana pipiens*. Using these analyses I tested hypotheses concerning how (or if) known geological events and key features of the species biology influenced the contemporary geographic and genetic distribution of *R. pipiens*. I assayed mitochondrial DNA variation from 389 individuals within 35 populations located throughout the species range. My a priori expectations for patterns and processes influencing the current genetic structure of *R. pipiens* were supported by the data. However, my analyses revealed specific aspects of *R. pipiens* evolutionary history that were unexpected. The phylogenetic analysis indicated that *R. pipiens* is split into two deeply divergent mtDNA groups, a western group and an eastern group, with the Mississippi River and Great Lakes region dividing the geographic ranges. Nested clade analysis indicated that the biological process most often invoked to explain the pattern of haplotype position is restricted gene flow with isolation by distance. Demographic analyses showed evidence of both historical bottlenecks and population expansions. Surprisingly, the genetic evidence indicated that the western region had significantly reduced levels of genetic diversity relative to the eastern region and that major range expansions occurred in both regions well before the most recent glacial retreat. This study provides a detailed history of how a widespread terrestrial vertebrate responded to episodic Pleistocene glacial events that occurred in North America.

Moreover, this study illustrates how complementary methods of data analysis can be used to disentangle recent and ancient effects on the genetic structure of a species.

INTRODUCTION

Understanding the evolutionary history of a species is a fundamental goal of evolutionary biology. In the past researchers typically used two main analytic approaches to elucidate the history of populations of a species from molecular data. They created bifurcating trees of populations, or they used $F_{st}$ to infer past vicariance events or to estimate $N_em$ (number of migrants per generation) under the assumption of drift-migration equilibrium in an island model. In the 1970s and 1980s, the application of the phylogeographic approach (Avise et al. 1979a, Avise et al. 1979b, Avise 2000) became the standard for measuring intraspecific variation. In the phylogeographic approach, phylogenetic affinities of haplotypes were overlain on geography, then the patterns were used to invoke demographical, behavioral, and historical details of the species involved. However, classic phylogeographic studies do not attempt to disentangle past events from contemporary processes. In the 1990's, the development of nested clade analysis (Templeton et al. 1987; Templeton et al. 1992; Templeton et al. 1993; Templeton et al. 1995) introduced a new way to analyze phylogeographic data. Nested clade analysis involves an overlay of geography on an estimated gene tree to measure the strength of any geography/phylogeny associations and to interpret the evolutionary processes responsible (Avise 2000). Moreover, this approach applies a statistical framework against which a null hypothesis of no association between geography and phylogeny must be rejected before evolutionary processes are invoked. Recently, the face of phylogeography has changed again. New techniques allow

for statistical tests of historical changes in population, region, or species demography, such as evidence for population expansions and bottlenecks. These techniques include, but are not limited to (i) mismatch distribution analysis (Slatkin and Hudson 1991; Rogers and Harpending 1992) which not only tests for the occurrence of demographic changes, but also dates the approximate time that these changes occurred and (ii) various tests initially developed to detect whether DNA polymorphisms are selectively neutral, but which additionally provide insight into population size changes (reviewed in Fu 1997). Together, phylogenetic, phylogeographic, and demographic techniques provide the tools necessary to understand how and when past processes have influenced current genetic composition of a species (Hewitt 2000).

These techniques allow testing of hypotheses concerning how (or if) known geological events influenced contemporary geographic and genetic distribution. Temperate North America provides a good example of a region that has a well-established history of drastic geologic events. Temperate North American flora and fauna have been greatly influenced by dynamic glacial oscillations (Hewitt 1993, Pielou 1991). Past studies have investigated the influence of glacial growth and contraction on the evolutionary history of many species (see summaries in Avise et al. 1998; Bernatchez and Wilson 1998). However, most of these studies have involved species with only limited ranges [examples include: the North American west (e.g. Conroy and Cook 2000; Johnson and Jordan 2000; Pook et al. 2000; Janzen et al. 2002), Pacific Northwest (e.g. Green et al 1996; Taylor et al. 1999;

Nielson et al. 2001), north (e.g. Turgeon and Bernatchez 2001), central states (e.g. Templeton et al. 1995; Ross 1999; Weisrock and Janzen 2000; Kreiser et al. 2001), and south (e.g. Burbrink et al. 2000)] and have therefore focused only on regional phylogeography. Recently, two studies have investigated wide-ranging species using a combination of phylogentic, phylogeographic, and demographic techniques. Both studies found that populations investigated had complex evolutionary histories that would not necessarily have been explained or detected by classical approaches alone (Bernatchez 2001, Althoff and Pellmyr 2002).

Here, I investigate the evolutionary history of the northern leopard frog, *Rana pipiens*. The leopard frog species complex has a storied history in evolutionary biology (see Hillis 1988). Various aspects of *R. pipiens* ecology have been well studied (Moore 1949; Dole 1971; Merrell 1977), but relatively few studies have looked at genetic variation within *Rana pipiens sensu stricto*. The northern leopard frog is broadly distributed, with a species range from the state of Washington to New York, and from Arizona to Hudson Bay. The majority of previous studies investigating geographic variation within leopard frogs have not differentiated among species within the complex (Moore 1949; Gillespie and Crenshaw 1966; Post and Pettus 1966; Salthe 1969), or only used limited sampling of northern leopard frogs (Dunlap and Platz 1981; Kimberling et al. 1996) and therefore provide little information about species-wide phylogeography.

My a priori hypotheses concerning the phylogeographic and demographic history of *R. pipiens* arise from two sources: (1) phylogeographic and population

genetic studies of anurans in general and (2) phylogeographic studies of other taxa within temperate North America. First, I predict that restricted gene flow plays an important role in the contemporary genetic composition of *R. pipiens* because studies have shown that isolation by distance and restricted movement are the main factors that influence anuran genetic structure (Yang et al. 1994; Driscoll 1998; Shaffer et al. 2000; Nielson et al. 2001; Rowe et al. 2000, Newman and Squire 2001; Monsen and Blouin, unpublished data). Second, I predict that within the framework of restricted gene flow, I will be able to detect the genetic signature of a demographic bottleneck and subsequent expansion caused by glacial advance and retreat (Hewitt 1996), including evidence of glacial refugia (Comps et al. 2001). This second hypothesis is supported by phylogeographic findings from other species found in temperate North America (e.g. Sage and Wolff 1986; Green et al. 1996; Bernatchez and Wilson 1998; Burbrink et al. 2000; Conroy and Cook 2000; Nielson et al. 2001, Starkey et al. 2003).

Support for these hypotheses would indicate that anuran evolution is influenced by Pleistocene glaciation in a manner similar to that of other North American species. Lack of support for these hypotheses would raise new questions concerning anuran evolution and glacial affects on small ectothermic vertebrates. Regardless of whether this study finds support for my hypotheses, it will provide a detailed history of how a widespread terrestrial vertebrate responded to the episodic Pleistocene glaciation events that occurred in North America. I will also provide one of the first examples of a study that uses the combined methodological

approach to elucidate a species' evolutionary history (see also Bernatchez 2001, Althoff and Pellmyr 2002).

To examine how the geological history of temperate North America influenced the contemporary genetic structure of *R. pipiens*, I sequenced part of the mtDNA NADH dehydrogenase subunit 1 (ND1) gene from multiple individuals within populations located throughout the species range. I used these data first to create an intraspecific phylogeny of *R. pipiens*, then used them to investigate past events and contemporary processes involved in shaping the present species genealogy. Additionally, I used mismatch distribution analysis (MDA) and neutrality tests to determine if and when any lineages underwent demographic fluctuations. Finally, I determined if my results support the above hypotheses concerning anuran biology and the effects of glaciation on northern leopard frogs.

## METHODS

### *Samples*

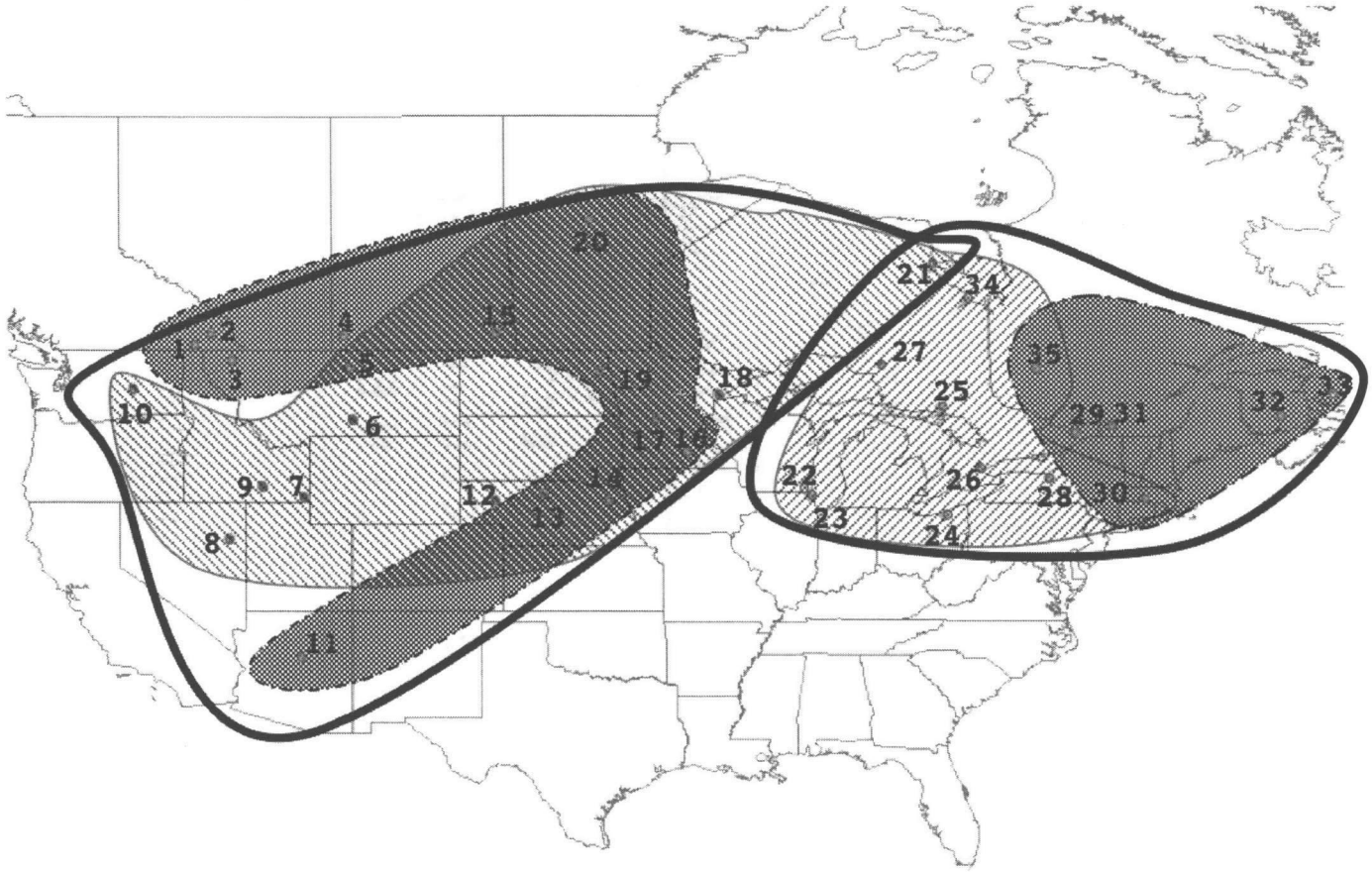
Samples of *R. pipiens* consisted primarily of toe clips collected during the summers of 1997 – 2001 and preserved by desiccation in 1.5 ml tubes filled with drie-rite desiccant or 95% ethanol. Some populations consisted of samples from the Canadian Museum of Nature (NMC), Ottawa, Canada, and one population (MON) was acquired from the Museum of Vertebrate Zoology's (MVZ; UC

Berkeley) frozen tissue stocks. A total of 389 samples representing 35 populations located throughout the species range (see Fig. 3.1, also see Appendix 3.1) were used in this study.

Total genomic DNA was extracted following a standard phenol/chloroform technique (Sambrook et al. 1989) or a standard chelex technique (Miller and Kapuscinski 1996). For mtDNA sequence analysis, I amplified the 5' region of the mtDNA ND1 gene, including the upstream tRNA –leucine, by polymerase chain reaction (PCR). I initially attempted to amplify all samples using primer MB77 (5'-TGGCAGAGCTTGGTTATGCAAAAGA-3') and primer MB145 (5'-CTAAGAGGAAGACACTCCAG-3'). However, some samples consisted of degraded DNA and would not amplify across this entire 644 bp region. Therefore I designed internal primers that split the 644 bp piece into two smaller pieces (MB77 x MB129; 5'-GGAAATGGGGTTCATATRATTARTG-3' and MB145 x MB128; 5'-RRCCCCCACYCTYFCCYTAAC-3'). Using these primers I was able to amplify all previously failed reactions. In all, a total of 644 bp of mtDNA were sequenced from all 389 samples used in this study.

PCR reactions were carried out in 25 μl reactions using standard buffer conditions, 1.5 mM $MgCl_2$, 10 mM each dNTP, 0.5 Units *Taq* DNA polymerase and approximately 100 ng DNA. After 3 min of initial denaturation, each of 35 cycles consisted of the following steps: 94°C for 45s, 47°C for 30s, and 72°C for 1 min, followed by a 7 min final extension. PCR product was visualized under UV light and samples were purified by using the MoBio Ultraclean PCR cleanup kit

FIG. 3.1. Map of North America showing the geographic distribution of *Rana pipiens* populations included in this study (identified by number located next to symbols denoting the latitudinal and longitudinal coordinates for each sample). For species range see Figure 6. Populations are grouped according to phylogeny. Thick dark line denotes the phylogenetic range of 4-step clades, indicating eastern and western lineages. Thin lines and shading within each 4-step clade indicate 3-step clades, showing further division within each lineage. Number of samples, occurrence and frequency of haplotypes in each location, and exact population location are provided in the Appendix.



(Salana Beach, CA) and sequenced by the Nevada Genomics Center (Reno, NV). A few initial samples were sequenced in both directions and used as reference sequences. The remaining samples were sequenced in only one direction with any ambiguous reads sequenced in the reverse direction for verification. All sequences were aligned with a type sequence and checked for correct base calls in SeqEd (1.0.3, Applied Biosystems, Inc.). After proofreading, all sequences were aligned using ClustalW (Higgins et al. 1994) and converted to nexus format for data analysis. Resulting sequences have been deposited in GenBank under accession numbers AF548568, AF548570 - AF548613, AY157644, AY157645.

*Phylogeny Reconstruction*

Phylogenetic trees of unique haplotypes were constructed using maximum likelihood (ML) optimality criterion implemented with PAUP* v. 4.0b10 (Swofford 2002) for each unique haplotype in the data set. For ML searches it is necessary to determine an appropriate model of sequence evolution (Swofford et al. 1996). I used likelihood ratio tests as implemented by Modeltest v. 3.0 (Posada and Crandall 1998) to infer the most likely model of sequence evolution for the data. Parameter space was searched for the best tree employing a heuristic search conducted with 10 random additions and TBR branch swapping. I utilized the nonparametric bootstrap method (100 pseudo-replicates with random addition at each iteration) to assess nodal support of the shortest tree (Felsenstein 1985). In

addition to the *R. pipiens* samples, an individual of both *R. blairi*, from Nebraska, and *R. utricularia*, from Florida, were also included in the phylogenetic analysis as outgroup species. These species were chosen as outgroups because they are *R. pipiens'* closest interspecific relatives (Hillis et al. 1983). Finally, time since sundering of deeply divergent groups was estimated using a molecular clock (0.69% sequence divergence/per lineage/MY). Although molecular clocks can only provide an approximate of age since divergence, I am confident that the clock I used is appropriate and provided a reasonable estimate in this species for three reasons. First, the clock was calibrated from geologic data for the mtDNA ND1 gene of an anuran (Macey et al. 1998). Second, similar rates of divergence have been shown in salamanders (Spolsky et al. 1992), lizards (Macey et al. 1998b), and snakes (Zamudio and Greene 1997). Third, this mtDNA clock yields dates of separation that are very similar to dates based on allozyme distances among Pacific Northwestern ranid species (Green 1986; Monsen and Blouin, unpublished data).

*Nested Clade Analyses*

To detect an association between geographic location and haplotype frequency and infer evolutionary processes that have influenced current genetic structure I implemented nested-clade analysis (NCA) as described by Templeton et al. (1995). First, I used the program TCS (Clement et al. 2000) to create an intraspecific network of haplotypes using the 95% statistical parsimony method of

Templeton et al. (1992). Second, the resulting network was converted into a nested design following the nesting algorithm of Templeton et al. (1987). Third, for clades that exhibited appropriate levels of genetic and geographic variation I used the program GeoDis (Posada et al. 2000) to calculate levels of significance for associations between haplotype and geographical location. Statistical testing of association between phylogenetic and geographic location was determined by random permutation tests of the data against a null hypothesis created from a random geographic distribution of all clades within each nesting level (generated using 10,000 permutations). Significance for the data was determined at the 5% level, and for those clades to which the null hypothesis was rejected I followed the inference key at http://inbio.byu.edu/Faculty/kac/crandall_lab/geodis.htm (updated October 2001) to infer biological causes for the observed associations.

*Demographic Analyses*

For comparisons of within-region genetic diversity, haplotype diversity (h) and nucleotide diversity ($\pi$) were calculated using Arlequin, version 2.0 (Schneider et al. 2000) and DNAsp version 3.53 (Rozas and Rozas 2001), respectively. Furthermore, I conducted a mismatch distribution analysis (MDA), constructing mismatch distributions and calculating the raggedness index of Harpending (1994), using Arlequin, to test for evidence of range expansion. One concern about using MDA for a group of subdivided populations, as found in this study, is that the

assumption of random mating is violated. However, Rogers (1995) showed that the theory behind MDA is robust and should approximately hold true even when populations are completely isolated. Moreover, when subdivided populations are linked by gene flow, you get an even better fit to the random mating approximation.

MDA did indeed indicate that range expansions had taken place in multiple clades, so I used the MDA age expansion parameter ($\tau$) to date the initiation of each range expansion (Rogers and Harpending 1992), assuming a 2 year generation for *R. pipiens* (Ryan 1953). Additionally, I calculated Tajima's D (Tajima 1989) and Fu's Fs test (Fu 1997) using Arlequin and Fu and Li's D*, Fu and Li's F* using DNAsp to confirm evidence of demographic expansions. These tests involve comparisons of different estimates of the parameter $\theta$, which is defined as $4N\mu$ for an autosomal locus, and $2N\mu$ for a haploid locus, where N is the effective population size and $\mu$ is the mutation rate per sequence per generation (Fu 1997).

## RESULTS

### *Phylogeny Reconstruction:*

Among the 389 ingroup specimens examined, I identified 51 polymorphic sites comprising 45 unique *R. pipiens* haplotypes (see Fig. 3.2, see Appendix 3.1). Modeltest selected the HKY+G model (Hasegawa et al. 1985) as the most likely

model of sequence evolution for the data, with gamma distribution shape parameter equal 0.2126 and a transition/transversion ratio of 5.3020. Figure 3.2 illustrates the branching topology of the ML tree. The most prominent feature of this tree involves the position of the outgroup species relative to two distinct haplotype groups, an eastern group and a western group. High bootstrap support groups together eastern haplotypes as a single monophyletic clade (Fig. 3.2). The odd shape of this tree likely originated from one of two processes. A western ancestor could have migrated eastward and given rise to the eastern lineage. Over time, owing to large population sizes and recurrent bottlenecks, the original sequence was retained unchanged in some western populations, while undergoing mutational changes in the eastern populations. This long-range dispersal hypothesis is supported by the nested clade analysis (see below). Alternately, the eastern and western haplotypes groups could have been split owing to some vicariant event. I used a Shimodaira-Hasegawa (SH) test to compare likelihoods of ML topologies of each of two trees representing extremely different potential trees given the data: one was the observed tree (Fig. 3.2), and one was a tree in which the western haplogroup arose from an ancestral node within the eastern haplogroup. These two trees where not significantly different ($p=0.122$), indicating that statistically, I cannot reject reciprocal monophyly among haplotype groups. However, for this vicariant event hypothesis to explain the observed tree topology, there would have needed to be a relatively large change in the rate of evolution among the two haplotype groups. Maximum sequence divergence (uncorrected) between eastern

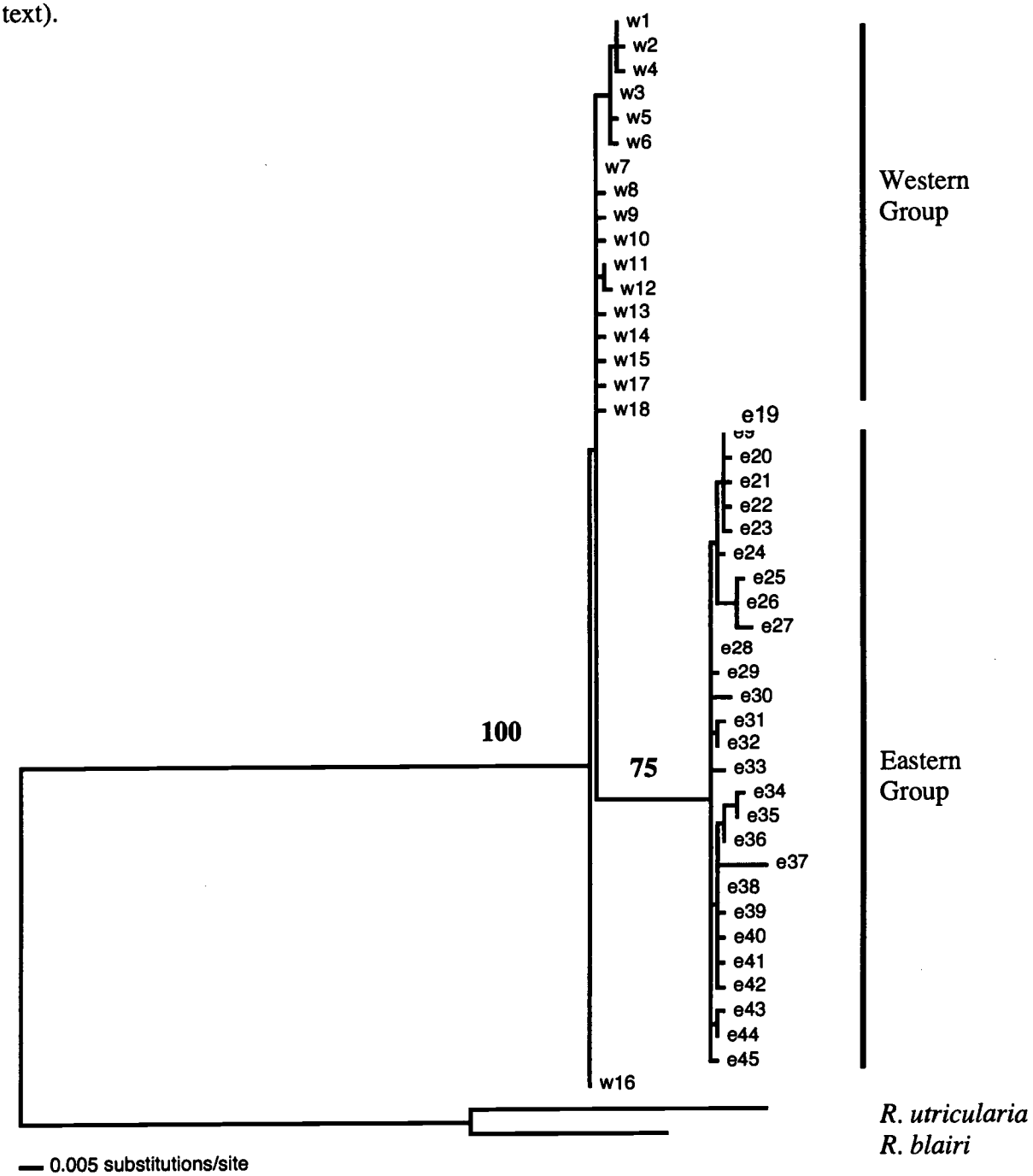
and western groups was 4.3%. Average uncorrected sequence divergence between the eastern and western groups was approximately 3%. Uncorrected sequence divergence was a maximum of 2.2% within the eastern group and 0.93% within the western group. The estimated age of haplotype group divergence between east and west populations of *R. pipiens* was about 2 MY (3% sequence divergence at 0.69% per lineage per MY).

Surprisingly, the Arizona population contained a single eastern haplotype (see Appendix). This haplotype is probably not from a native frog but rather, was likely a liberated pet or laboratory frog. Laboratory frogs are almost always collected in the eastern part of the species range (Hoffman and Blouin, unpublished data). For this reason, the individual with the eastern haplotype from Arizona was removed from subsequent intra-regional analyses.

*Nested Clade Analysis*

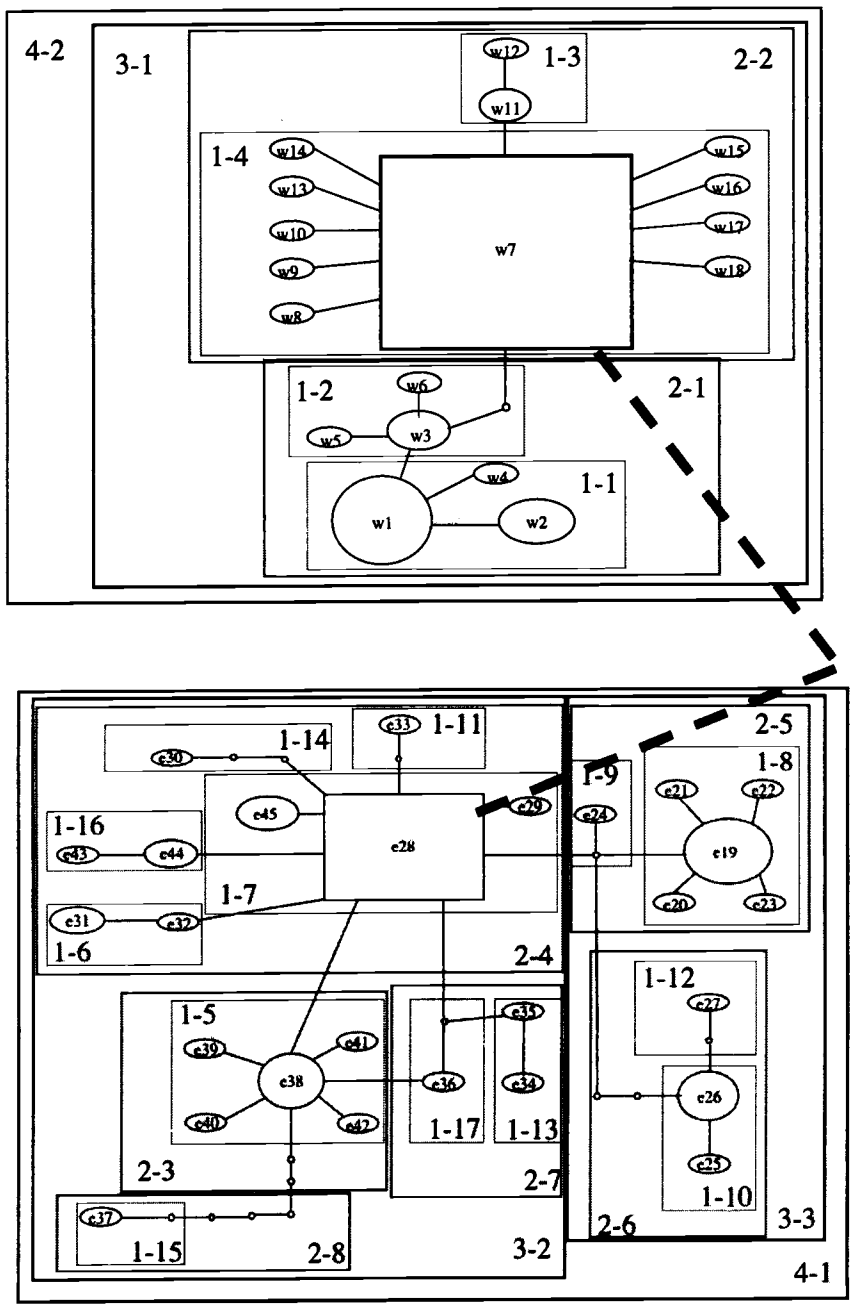
Figure 3.3 shows the intraspecific network and nesting design obtained using the nesting algorithm given in Templeton et al. (1987). One reticulation caused by an inferred haplotype connecting haplotypes w1 and w18 was removed based on the criteria of Posada and Crandall (2001) for determining the most

FIG. 3.2. Single tree retained from maximum-likelihood analysis relating the two major *R. pipiens* haplotype groups. Relationships among groups and bootstrap values (greater than 75%; above line) are derived from the 644 bp of the mtDNA ND1 gene. Alphanumeric labels following tree tips designate haplotype name. Within-population frequency of haplotypes is provided in the Appendix. This tree is rooted by two outgroup species, *R. blair*i and *R. utricularia*. Location of the eastern haplotypes nested within a backbone of western haplotypes suggests that the eastern group is derived from the western group. However, I cannot reject the hypothesis of reciprocal monophyly for the east and west haplotype groups (see text).



plausible of alternative networks. A second reticulation (connecting haplotypes e28, e38, and e36) could not be removed and was therefore nested in accordance to the nesting algorithm. Third and fourth level nesting (Fig. 3.1) illustrates the break between eastern and western haplotype groups as well as sub-structuring within each region. TCS could not link eastern and western haplotype groups within the limits of 95% statistical parsimony. Ninety-five percent statistical parsimony was accepted only up to 10 mutational steps, but the east-west connection required 16 mutational steps, indicating that these connections are beyond the 95% confidence limits of parsimonious connections. However, two sources of evidence suggest that the east-west connection does indeed link clades 1-7 and 1-4 as shown in Fig. 3.2: (i) in TCS, when the number of maximum connection steps allowed is changed to 16 the program links 1-7 and 1-4 (although not with 95% confidence), and (ii) clades 1-7 and 1-4 contain haplotypes with the highest outgroup weights (Castelloe and Templeton 1994) within each region and thus are most likely connected together according to predictions of coalescent theory applied to intraspecific networks (Posada and Crandall 2001). Outgroup weight calculations indicated that clade 1-4 contained the most ancestral haplotype (w7), therefore at the highest nesting level clade 4-2 (which contains clade 1-4) was assumed to be interior (Posada and Crandall 2001). The polarity of the west being ancestral to the east concurs with the intraspecific phylogeny where the western haplotype group is more similar to outgroup species than the eastern haplotype group. The null

FIG. 3.3. Statistical parsimony (95%) network for 45 mtDNA haplotypes identified for *R. pipiens*. The network is grouped into nesting clades. Each line in the network represents a single nucleotide substitution. Small circles indicate undetected intermediate haplotype states. The relative size of the boxes and ovals (at the haplotype level) represent the frequency of these haplotypes (boxes around haplotypes denote those haplotypes with the highest outgroup weight). The dotted line connecting haplotype w7 to e28 was not identified within the limits of 95% statistical parsimony, but is supported by other data (see text). The boxes indicate nesting level (e.g. 1-4, nesting level one, grouping number four). Alphanumeric haplotype names correspond to those in Figure 3.2 and the Appendix 3.1.



hypothesis of no association between geographic location and haplotype position within a clade was rejected for 15 clades, showing that both population structure and population history played important roles in determining the current position of mtDNA haplotypes (see Fig. 4). The biological processes inferred to cause the significant associations for all of these clades are described in Table 1. The biological process most often invoked to explain the pattern of haplotype position observed for *R. pipiens* is restricted gene flow with isolation by distance. However, allopatric fragmentation, range expansion, or long distance colonization were also inferred for certain clades.

*Demographic Analyses*

Levels of haplotype diversity and nucleotide diversity are not equal between geographic regions. Haplotype diversity (h) and nucleotide diversity ($\pi$) are significantly higher in the east than in the west (see Table 2). Furthermore, the average number of nucleotide differences is almost twice as great in the east (3.293) as in the west (1.957).

Mismatch distributions for each of the four clades for which NCA indicated range expansion (analyzed separately) and for the entire west region fit the expected unimodal model of sudden expansion (see Table 3, see Fig. 5). Additionally, for each clade analyzed separately the raggedness index was low (see Table 3) indicating a smooth distribution and thus further evidence of population expansion

(Harpending 1994). Therefore, I can use the observed values of the MDA age expansion parameter ($\tau$) to estimate the time the expansion began for clades initially identified by NCA as undergoing range expansion. This was done by solving for $\tau$, from $\tau=2ut$ (Rogers and Harpending 1992), where u is the sum of per-nucleotide mutation rates in the region of DNA under study and t is time in generations. Values of $\tau$ were nearly identical in the western clades (clade 1-4 $\tau=3.022$, clade 4-2 $\tau=3.61$), but different and older in the eastern clades (clade 3-3 $\tau=5.02$, clade 4-1 $\tau=3.68$). When $\tau$ is converted to years since the expansion, range expansions are dated from 330,000 years ago for clade 1-4 (with 95% confidence intervals ranging from 50,000 – 710,000) to 550,000 years ago for clade 3-3 (with 95% confidence intervals ranging from 146,000 – 1,127,000) (see Table 3).

Significance of neutrality tests varied depending on statistic and region. Within the western region Fu and Li's D* and Fu and Li's F* were significant and Fu's Fs was marginally significant, however, Tajima's D was not significant (see Table 2). Within the eastern lineage, Fu's Fs was significant, but Tajima's D, Fu and Li's D* and Fu and Li's F* were not significant (see Table 2). Nevertheless, nominal values of all test statistics within both regions had negative values, again suggesting that demographic expansions had occurred.

FIG. 3.4. Results of nested clade analysis for (A) western and (B) eastern populations of *R. pipiens*. Haplotype alphanumeric designation is given at the top and are boxed together to reflect one-step clades. These one-step clades are combined into higher level nesting groups through boxes lower down the figure. Nested design and clade designations are given in Figure 3. Shaded boxes represent interior clades, tip clades are unshaded. The clade distance ($D_c$) and nested-clade distance ($D_n$) are given below each clade designation. If the clade contains both tip and interior groups (I – T), the contrast between them is given for both $D_c$ and $D_n$ in the bottom two lines in the box. Significance was determined at the 5% level. Significantly small values are followed by "S", significantly large values are followed by "L".

FIG. 4a.

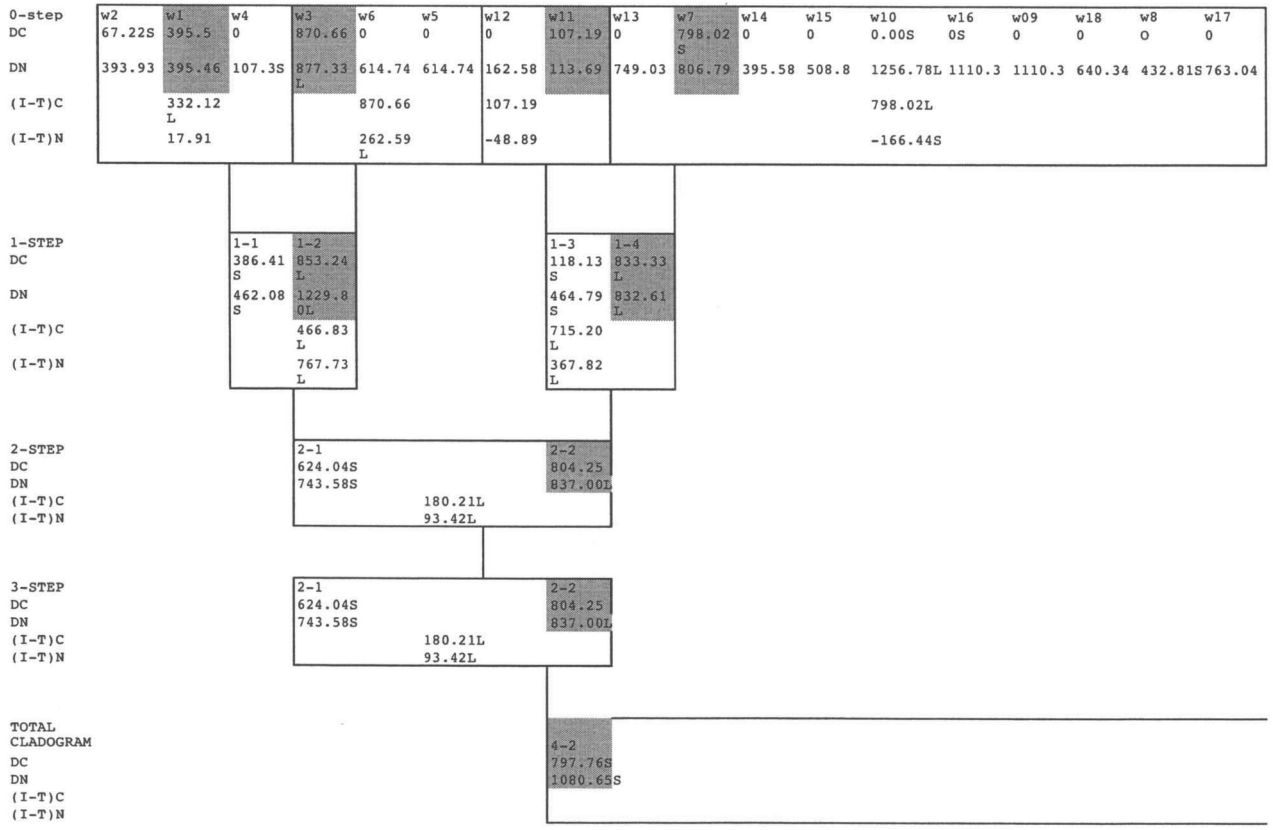

0-step
DC
DN
(I-T)C
(I-T)N
w2 w1 w4 w3 w6 w5 w12 w11 w13 w7 w14 w15 w10 w16 w09 w18 w8 w17
67.22S 395.5 0 870.66 0 0 0 107.19 0 798.02S 0 0 0.00S 0S 0 0 0 0
393.93 395.46 107.3S 877.33L 614.74 614.74 162.58 113.69 749.03 806.79 395.58 508.8 1256.78L 1110.3 1110.3 640.34 432.81S 763.04
332.12L 870.66 107.19 798.02L
17.91 262.59L -48.89 -166.44S
1-STEP
DC
DN
(I-T)C
(I-T)N
1-1 1-2 1-3 1-4
386.41S 853.24L 118.13S 833.33L
462.08S 1229.80L 464.79S 832.61L
466.83L 715.20L
767.73L 367.82L
2-STEP
DC
DN
(I-T)C
(I-T)N
2-1 2-2
624.04S 804.25
743.58S 837.00L
180.21L
93.42L
3-STEP
DC
DN
(I-T)C
(I-T)N
2-1 2-2
624.04S 804.25
743.58S 837.00L
180.21L
93.42L
TOTAL
CLADOGRAM
DC
DN
(I-T)C
(I-T)N
4-2
797.76S
1080.65S

FIG 4b.

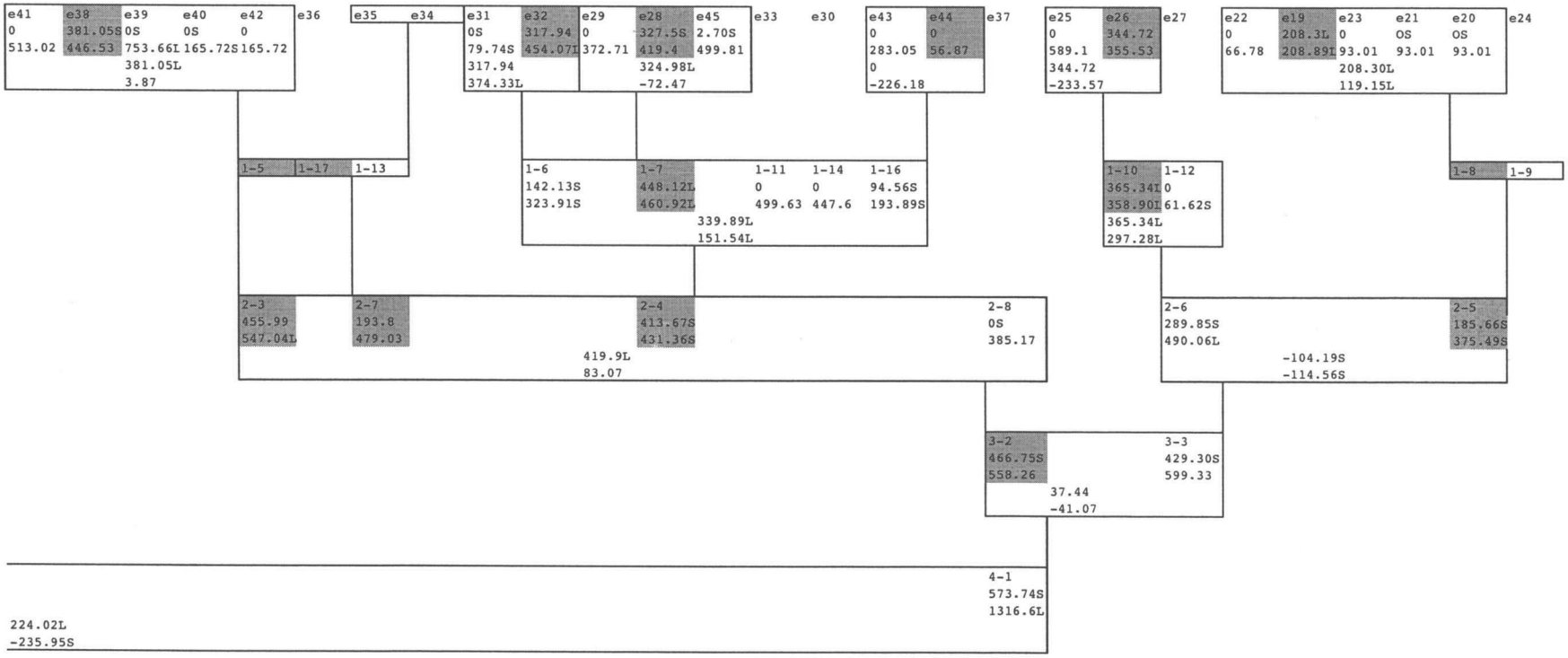

e41 e38 e39 e40 e42 e36
0 381.05S 0S 0S 0
513.02 446.53 753.66I 165.72S 165.72
381.05L
3.87
e35 e34
e31 e32 e29 e28 e45 e33 e30
0S 317.94 0 327.56 2.70S
79.74S 454.07I 372.71 419.4 499.81
317.94 324.98L
374.33L -72.47
e43 e44 e37
0 0
283.05 56.87
0
-226.18
e25 e26 e27
0 344.72
589.1 355.53
344.72
-233.57
e22 e19 e23 e21 e20 e24
0 208.3L 0 OS OS
66.78 208.89I 93.01 93.01 93.01
208.30L
119.15L
1-5 1-17 1-13
1-6 1-7 1-11 1-14 1-16
142.13S 448.12L 0 0 94.56S
323.91S 460.92L 499.63 447.6 193.89S
339.89L
151.54L
1-10 1-12
365.34I 0
358.90I 61.62S
365.34L
297.28L
1-8 1-9
2-3 2-7 2-4 2-8
455.99 193.8 413.67S 0S
547.04L 479.03 431.36S 385.17
419.9L
83.07
2-6 2-5
289.85S 185.66S
490.06L 375.49S
-104.19S
-114.56S
3-2 3-3
466.75S 429.30S
558.26 599.33
37.44
-41.07
4-1
573.74S
1316.6L
224.02L
-235.95S

TABLE 3.1. Inference for all clades showing significant association from NCA results given in Fig. 4 from key given from http://inbio.byu.edu/Faculty/kac/crandall_lab/geodis.htm (October 2001).

| Clade | Chain of Inference | Inference |
|---|---|---|
| Zero-step clades nested in 1-1 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| Zero-step clades nested in 1-4 | 1-2-3-5-6; 7 -YES | Either range expansion/colonization or restricted gene flow/dispersal with some long distance dispersal |
| Zero-step clades nested in 1-5 | 1-2-3-5-15-16-YES | Allopatric fragmentation |
| Zero-step clades nested in 1-6 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| Zero-step clades nested in 1-7 | 1-2-3-4-9-10-Yes | Allopatric fragmentation |
| Zero-step clades nested in 1-8 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| One-step clades nested in 2-1 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| One-step clades nested in 2-2 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| One-step clades nested in 2-4 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| One-step clades nested in 2-6 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| Two-step clades nested in 3-1 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| Two-step clades nested in 3-2 | 1-2-3-4-NO | Restricted gene flow with isolation by distance |
| Two-step clades nested in 3-3 | 1-2-11-12-13-14-NO | Either contiguous range expansion or long distance colonization |
| Three-step clades nested in 4-1 | 1-2-11-12-NO | Contiguous range expansion |
| Four-step clades nested in entire cladogram | 1-2-11-12-13-YES | Long distance colonization |

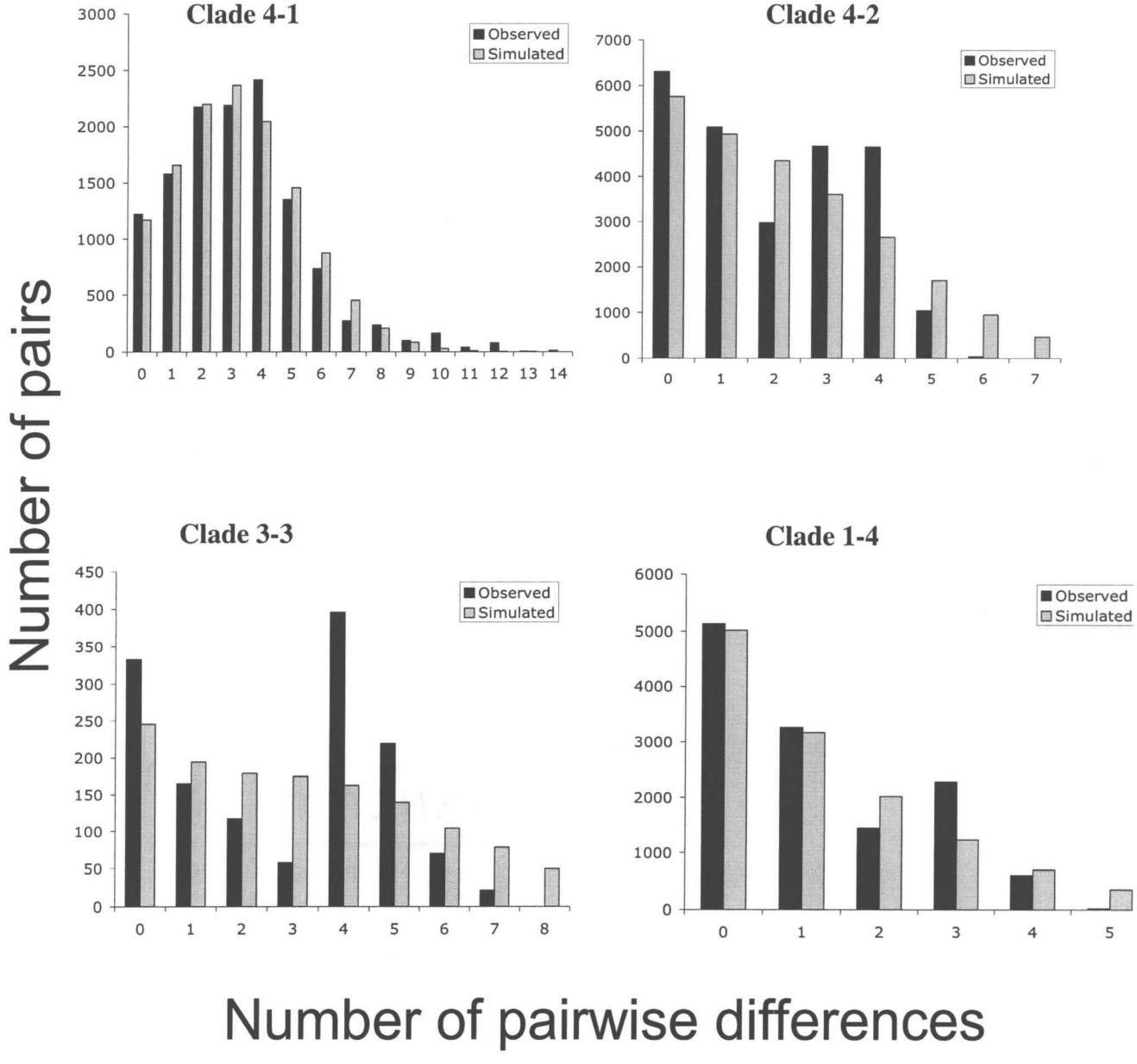
TABLE 3.2. Results of demographic parameters including number of populations, h, $\pi$, Fu's Fs, Tajima's D, Fu and Li's D* and F* (95% CI and p-values given where applicable), grouped by geographic region.

| Region | Number of populations | Haplotype Diversity (h) (95% CI) | Nucleotide Diversity ($\pi$) (95% CI) | Fu's Fs (p-value) | Tajima's D (p-value) | Fu and Li's D* (p-value) | Fu and Li's F* (p-value) |
|---|---|---|---|---|---|---|---|
| West (Clade 4-2) | 21 | 0.745 (0.045) | 0.003 (± 0.00022) | -5.277 (0.057) | -0.664 (0.27) | -3.18 (<0.05) | -2.65 (<0.05) |
| East (Clade 4-1) | 14 | 0.903 (0.020) | 0.005 (± 0.00063) | -9.60 (0.012) | -1.43 (0.075) | -1.17 (>0.10) | -1.54 (>0.10) |

TABLE 3.3. Observed (S) and 95% Confidence Intervals (CI) of simulated number of polymorphic sites, mean number of pairwise differences (95% CI), demographic parameters (95% CI) within clades with *a priori* evidence (from NCA) of range expansion. $P(SSD_{obs})$ and $P(Rag_{obs})$ measure the probability of deviation from a hypothesis of sudden expansion according to the mismatch distribution and raggedness index respectively. Years since expansion measures the approximate time since the expansion began estimated from $\tau$, the age expansion parameter (95% CI).

| Clade | S (95% CI) | Mean number of differences | $\tau$ | $\theta_0$ | $\theta_1$ | $P(SSD_{obs})$ | Raggedness | $P(Rag_{obs})$ | Years since expansion initiation (MYA) (95% CI) |
|---|---|---|---|---|---|---|---|---|---|
| 1-4 | 15 (3-15) | 1.22 (0.13-2.87) | 3.022 (0.48-6.37) | 0.004 (0–0.882) | 1.563 (0.158–2116.6) | 0.62 | 0.0659 | 0.73 | 0.3358 (0.05 – 0.71) |
| 4-2 | 16 (9-25) | 1.98 (0.87-3.69) | 3.61 (0.686-6.74) | 0.001 (0-1.79) | 3.435 (1.029-2659.4) | 0.51 | 0.037 | 0.70 | 0.401 0.076 – 0.749 |
| 3-3 | 14 (8-26) | 2.77 (1.05-5.41) | 5.02 (1.315-10.145) | 0.004 (0-4.257) | 5.064 (1.395-1986.3) | 0.10 | 0.108 | 0.13 | 0.550 (0.146 – 1.127) |
| 4-1 | 35 (27-51) | 3.28 (1.81-4.61) | 3.68 (1.25-5.39) | 0.002 (0-2.34) | 12.177 (5.736-3900.9) | 0.79 | 0.015 | 0.90 | 0.408 0.139 – 0.599 |

FIG. 3.5. Histograms of pairwise number of mutational differences of ND1 sequences for all clades identified by NCA to have undergone range expansions (and the entire western clade). Dark shading indicates observed values, light shading indicates the model fitted to the data. Significance values for bot goodness of fit between observed and simulated mismatch and the raggedness index indicate that each histogram does not deviate from expectations of expansion (see Table 3).



# DISCUSSION

## *Evolutionary history of the northern leopard frog*

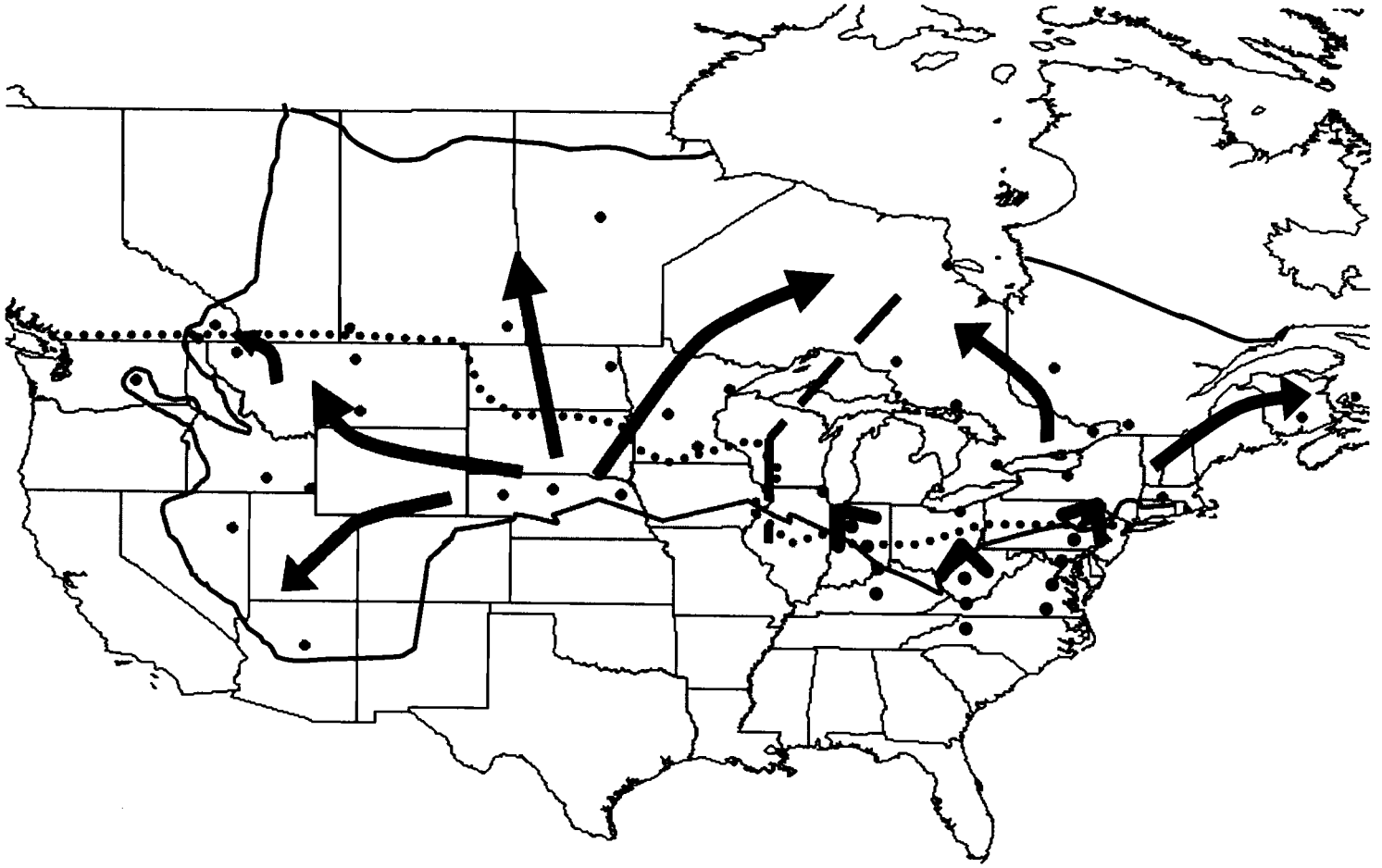
The phylogenetic analysis indicated that *Rana pipiens* is split into two deeply divergent mtDNA groups, a western group and an eastern group (Fig. 2), with the Mississippi River and Great Lakes region dividing the geographic ranges (Fig. 1, Fig. 6). Molecular-clock estimates of the anuran mtDNA ND1 gene indicate that east and west haplotype groups have been differentiated for approximately 2 million years. This would correspond to a leopard frog ancestor radiating across northern North America and subsequently differentiating during the Pliocene, a time when few North American anuran fossils exist, but believed to be a time of great species radiation in North American amphibians (Holman 1995). Geographically, these two haplotype groups are almost entirely disjunct (Fig. 1). Although I do not know how the eastern populations were founded, they likely spread from a few founders either crossing the Mississippi River or by spreading north of the great lakes. Regardless of the founding processes, glacial events and the Mississippi River kept eastern and western populations isolated by not allowing high levels of recurrent gene-flow.

Only one population, number 21 (located near Attawapiskat, Ontario), indicates an area of geographic convergence of east and west clades. This population is located

north of the great lakes region and likely contains both clades due to secondary contact during the current interglacial period. Thus, it represents the apex of post-glacial eastward expansion of the western haplotype and westward expansion of the eastern haplotype. The level of mtDNA genetic variation between eastern and western groups is relatively high for intraspecific comparisons. In fact, this amount of genetic variation is comparable to that found between some recognized species of ranid frogs such as *R. pretiosa-R. lutieventris* – about 3% (Monsen and Blouin, unpublished data), *R. yavapaiensis-R. onca* – about 4.7% (Jaeger et al. 2001), and *R. blairi-R. berlanderi* – about 4.9% (Jaeger et al. 2001). However, I do not recommend splitting *R. pipiens* into two distinct species because I only have evidence based on mtDNA. While the mtDNA divergence may be comparable to that found between other ranid species, studies have shown that mtDNA and other measures used to split species (e.g. nuclear markers, morphology, or behavior) are not always concordant (Nyakaana and Arctander 1999; Buonaccorsi et al., 1999; Franck et al., 2001; Monsen and Blouin, unpublished data). Nonetheless, further work on the taxonomic status of the two *R. pipiens* haplotype groups is warranted.

Application of NCA to the ND1 gene of *R. pipiens* adds further insight into the evolutionary history of the species. First, at the highest nesting level (the four-step clade), representing the oldest significant event, NCA identifies the origin of the eastern clade as a long distance colonization event originating from the western clade (see Table 1). This conclusion is supported by the

FIG. 3.6. Map showing the current geographic distribution of *R. pipiens* from Conant and Collins (1991; thin line) and major dispersal patterns as identified in this study. Points on map indicate sampling locations (See Figure 1). The thin dotted line running east to west approximates the southernmost extent of the Wisconsin glaciation. Solid arrows indicate dispersal patterns identified within the current species range. Dotted arrows indicate patterns of dispersal from past glacial refugia (as indicated by fragmentation events in nested clade analysis). The large dashed line symbolizes the geographic divide between the East/West genetic split.



topology of the ML tree (see Fig. 2), which is consistent with a basal western haplotype group giving rise to a more derived eastern haplotype group. Second, because within-region levels of sequence divergence were low, NCA was useful for detecting and interpreting significant relationships within these regions. Throughout the east and west regions, a consistent pattern of restricted gene flow is detected at all but the highest nesting levels. However, other processes also played significant roles in both regions. In the eastern region, allopatric fragmentation events from the main eastern lineage were identified in both Illinois (zero-step clades nested in clade 1-7) and Ohio (zero-step clades nested in clade 1-5 and clade 1-7) (see Table 1). Moreover, NCA identified that two range expansion events occurred in the eastern lineage, first across the east and into the northwest part of the eastern range (clade 4-1) and subsequently into the northeast (clade 3-3) (see Table 1). In the western region, restricted gene flow was the most influential force, although range expansions (clade 4-1) also helped shape extant haplotype locations (see Table 1).

Demographic analyses show evidence of both historical bottlenecks and population expansions that are likely due to the oscillation of glacial advance and retreat during the Pleistocene epoch. A comparison of levels of nucleotide diversity between the eastern and western clade shows the striking pattern of reduced levels of genetic variation in the west (Table 2). Although a selective sweep could have caused patterns similar to those found here, the most parsimonious explanation for the reduced levels of nucleotide diversity in the west is a demographic bottleneck (see below). Following this bottleneck,

subsequent range expansion led to the current distribution of northern leopard frogs. Although nucleotide diversity in the east is not as reduced as in the west, MDA and neutrality tests concurred with NCA that recent range expansions have occurred in this region as well. Graphs of mismatch distributions from all clades where NCA indicated range expansions occurred, and from the entire western region, were consistent with the unimodal model of sudden expansion for pairwise nucleotide differences (Rogers and Harpending 1992; see Table 3). Furthermore, I used the NCA analysis and patterns of genetic variation to predict patterns of range expansion from areas of glacial refugia (Fig. 6). The bottlenecks inherent in the data must have occurred prior to current expansion events because NCA, MDA and neutrality tests indicate that the most recent event influencing current genetic structure in both the eastern and western haplotype groups is population range expansion. Therefore, I used MDA to date the approximate time since the initiation of expansion. Average dates of expansion are similar within the eastern haplotype group, within the western haplotype group, and between the east and west group (see Table 3) indicating that major demographic events likely occurred species wide approximately 300,000 – 500,000 years ago (likely towards the later date since $\tau$ usually leads to an underestimation of age of expansion within the 95% confidence interval (Schneider and Excoffier 1999)). These demographic fluctuations likely correspond to widespread contraction and expansion from glacial refugia because these events were nearly ubiquitous among other vertebrate populations during the Pleistocene (Holman 1995).

A selective sweep could also explain the difference in genetic variation between eastern and western regions. However, another species complex (*Chrysemys*), which has a range similar to that of *R. pipiens*, exhibits the same patterns of eastern-versus-western divergence and reduced genetic variation in populations from the western United States (Starkey et al. 2003). Therefore, although a selective sweep could give similar results, it is more likely that geologic conditions, such as repeated glacial oscillations, left the signature of genetic bottlenecks in both species in the west.

*Do these phylogenetic and phylogeographic patterns fit the expectations?*

My a priori expectations for patterns and processes influencing the current genetic structure of *R. pipiens* consisted of two hypotheses: that restricted gene flow plays an important role in the contemporary genetic composition of *R. pipiens* and that I will be able to detect a genetic signature of glacially-instigated demographic bottlenecks and post-glacial expansions. Both of these predictions are supported by the data here. NCA clearly indicates that isolation by distance is the main process influencing contemporary genetic composition of *R. pipiens* (see Table 1). Additionally, both NCA and demographic analyses indicate a significant influence of population extinction and expansion. Glacial refugia, as indicated by high allelic richness and/or geographic regions containing multiple unique haplotypes, can be identified in both east and west region. Furthermore, comparative phylogeography of other species confirms that the Mississippi River is a major barrier to dispersal for

many species of small mammals, fishes, amphibians, and reptiles (Blair 1965; Lansman et al. 1983; Burbrink et al. 2000 and references therein, Starkey et al. 2003) in addition to *R. pipiens*.

However, my analyses revealed specific aspects of *R. pipiens* evolutionary history that were unexpected. For example: The intraspecific phylogeny for *R. pipiens* exhibited an interesting topology (Fig. 2) with both an eastern and western haplotype group. Moreover, the western haplotype group contained the haplotype that represented the node that split into the eastern and western groups. Two processes could explain this topology. First, a vicariant event could have split eastern and western populations and the rate of evolution in the west could have nearly stopped, while the eastern region underwent great genetic change. Alternately, the eastern lineage could have arisen from a more basal western haplotype group. NCA supports the second hypothesis because haplotype w7 contains the largest outgroup weight. Therefore NCA concludes that the eastern haplotype group was derived from the western haplotype group via long distance colonization (Table 1). This indicates that eastern and western groups did not arise via vicariant events, but rather the eastern group originated through a dispersal event from the west. This pattern is not unique. Other species with pre-glacial northern ranges also show evidence of long-range dispersal events causing eastern and western races (e.g. Wilson et al. 1996; Bernatchez 1997; Turgeon and Bernatchez 2001). A second unexpected result was that the western group of *R. pipiens* had significantly reduced levels of genetic variation relative to the eastern group. This indicates that population contraction affected the west more severely than the east. This would occur if

the number of glacial refugia differed between the east and the west regions, such that the east had more refugia, each of which maintained a different subset of the regional variation. This hypothesis is supported by the data, which suggest only one western population, number 14 (in eastern Nebraska) served as a glacial refugium, whereas five eastern populations (in Illinois, Ohio, Ontario, New York, and Massachusetts) appear to have served as glacial refugia based on high allelic diversity and/or multiple unique haplotypes (see Figure 6, see Appendix). Additionally, the demographic expansions identified by NCA, MDA, and neutrality tests occurred much earlier than expected. Contrary to my expectations, these expansions are not genetic signatures of the most recent glacial retreat (circa 15,000 years ago). However, the effects of less important demographic changes may be masked by the most influential event in recent history (Rogers 1995). Therefore, if population genetic variation was greatly reduced 300,000 – 500,000 years ago, then MDA will not pick up the signature of more recent, but less significant, bottlenecks.

*Benefits of using multiple analyses to elucidate evolutionary history*

My study demonstrates the benefit of using multiple analyses to elucidate a species' natural history from genetic data. In this study, I used three main approaches: phylogeny reconstructions, phylogeography (NCA), and demographic analyses (MDA). No single analysis would have provided a history as complete as did the complement of these techniques together. For example, the ML tree was informative concerning broad genetic patterns,

especially with regards to positions of outgroup taxa. However, the ML tree by itself would have only showed that there were distinct eastern and western groups. It would have provided little information about relationships among haplotypes within each group (owing to short branch lengths), or about the processes that generated that structure. To determine the relationship among haplotypes and their population frequencies within each region, I had to use an intraspecific haplotype network. NCA was useful in identifying the likely processes causing the current distribution of haplotypes. MDA and the other neutrality tests enabled an independent test of the data to determine if certain conclusions from the NCA (such as range expansions) were supported. Yet, demographic tests alone can prove misleading because various microevolutionary forces leave similar genetic fingerprints (e.g. expansion and selection). Similarly, NCA is not always accurate in predicting process from genetic patterns (Knowles and Maddison 2002). But in this case the concordant results of the different methods used give me confidence in each individual method. As data continue to indicate that many species' genetic structures are not in a stable equilibrium, it is increasingly important to use a suite of techniques to infer an accurate picture of evolutionary history.

## CONCLUSIONS

The results of this study assist in our understanding of how evolutionary events, predominately Pleistocene glacial oscillations, influenced a broad ranging, ectothermic vertebrate in temperate North America. However, I would

have received an incomplete history had I not used a full suite of phylogeographic tools, including phylogeny reconstruction, nested clade analysis, mismatch distribution analysis, and various neutrality tests. Using these analyses, I was able to determine that *R. pipiens* has a complex evolutionary history involving deeply divergent clades that originated via long distance dispersal in addition to experiencing allopatric fragmentation events, population bottlenecks and population expansions. Genetic evidence indicates major range expansions occurred well before the most recent glacial retreat. Moreover, this study identified how both eastern and western haplotype groups of *R. pipiens* were differentially influenced by glacial expansion and retreat as evidenced by significant variation in genetic diversity between groups. Finally, this study illustrates how complementary methods of data analysis can be used to disentangle recent and ancient effects on the genetic structure of a species.

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APPENDIX 3.1. List of all populations and the state or province in which they occur, haplotype (and frequency) in each population, latitude, longitude, and museum catalog numbers (if applicable; NMC=Canadian Museum of Nature, Ottawa; MVZ=Museum of Vertebrate Zoology, UC Berkeley).

| Population | State or Province of population | n | Haplotype (frequency) | Latitude | Longitude | Museum catalog number |
|---|---|---|---|---|---|---|
| 1 | BC | 12 | W02 (1.00) | 49.05 | -116.502 | N/A |
| 2 | BC | 15 | W02 (1.00) | 49.711 | -115.739 | NMC16081-1 – 16081-15 |
| 3 | MT | 12 | W01 (0.50) W02 (0.50) | 48.450 | -114.680 | N/A |
| 4 | SASK | 10 | W01 (0.80) W04 (0.20) | 49.658 | -109.494 | NMC16089-1 – 16089-10 |
| 5 | MT | 6 | W01 (1.00) | 48.159 | -109.2003 | N/A |
| 6 | MT | 9 | W01 | 45.693 | -108.984 | MVZ 501161 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | (0.78) | | | |
| | | | W07 | | | |
| | | | (0.22) | | | |
| 7 | ID | 12 | W07 | 42.139 | -111.262 | N/A |
| | | | (0.92) | | | |
| | | | W13 | | | |
| | | | (0.08) | | | |
| 8 | NV | 12 | W07 | 40.300 | -114.830 | N/A |
| | | | (0.67) | | | |
| | | | W09 | | | |
| | | | (0.08) | | | |
| | | | W16 | | | |
| | | | (0.25) | | | |
| 9 | ID | 12 | W07 | 42.620 | -113.284 | N/A |
| | | | (1.00) | | | |
| 10 | WA | 12 | W07 | 47.076 | -119.354 | N/A |
| | | | (0.25) | | | |
| | | | W10 | | | |
| | | | (0.75) | | | |
| 11 | AZ | 10 | W3 | 34.830 | -111.510 | N/A |
| | | | (0.90) | | | |
| | | | E19 | | | |
| | | | (0.10) | | | |
| 12 | NE | 12 | WO7 | 41.939 | -102.387 | N/A |
| | | | (0.92) | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | W14 | | | |
| | | | (0.08) | | | |
| 13 | NE | 12 | W03 | 42.237 | -100.088 | N/A |
| | | | (0.08) | | | |
| | | | W07 | | | |
| | | | (0.17) | | | |
| | | | W08 | | | |
| | | | (0.17) | | | |
| | | | W11 | | | |
| | | | (0.58) | | | |
| 14 | NE | 12 | W03 | 42.001 | -97.0064 | N/A |
| | | | (0.08) | | | |
| | | | W05 | | | |
| | | | (0.08) | | | |
| | | | W06 | | | |
| | | | (0.08) | | | |
| | | | W07 | | | |
| | | | (0.42) | | | |
| | | | W11 | | | |
| | | | (0.25) | | | |
| | | | W12 | | | |
| | | | (0.08) | | | |
| 15 | SASK | 10 | W01 | 49.817 | -102.267 | NMC 24276- |
| | | | (0.20) | | | 1 – 24276-10 |
| | | | W07 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | (0.80 | | | |
| 16 | MN | 12 | W03 | 44.215 | -93.593 | N/A |
| | | | (0.17) | | | |
| | | | W07 | | | |
| | | | (0.67) | | | |
| | | | W17 | | | |
| | | | (0.17) | | | |
| 17 | MN | 12 | W03 | 45.710 | -94.893 | N/A |
| | | | (0.42) | | | |
| | | | W07 | | | |
| | | | (0.50) | | | |
| | | | W18 | | | |
| | | | (0.08) | | | |
| 18 | MN | 12 | W07 | 46.921 | -92.156 | N/A |
| | | | (1.00) | | | |
| 19 | MN | 12 | W01 | 47.938 | -97.506 | N/A |
| | | | (0.42) | | | |
| | | | W07 | | | |
| | | | (0.50) | | | |
| | | | W15 | | | |
| | | | (0.08) | | | |
| 20 | MAN | 10 | W01 | 55.030 | -98.040 | NMC 18594- |
| | | | (0.40) | | | 1 – 18594-7; |
| | | | W07 | | | 20127-1,2,4,5 |
| | | | (0.60) | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 21 | ONT | 5 | W07 (0.60) E38 (0.40) | 52.875 | -82.292 | NMC 23821; 23824; 23825; 25110-1,2 |
| 22 | IL | 10 | E39 (0.40) E45 (0.60) | 42.254 | -87.887 | N/A |
| 23 | IL | 11 | E33 (0.18) E45 (0.82) | 42.204 | -87.888 | N/A |
| 24 | OH | 12 | E29 (0.08) E31 (0.83) E36 (0.08) | 41.362 | -81.602 | N/A |
| 25 | ONT | 12 | E28 (0.92) E43 (0.08) | 46.275 | -81.871 | NMC 23839-1 – 23839-12 |
| 26 | ONT | 12 | E28 (0.17) E32 | 43.518 | -79.997 | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | (0.08) | | | |
| | | | E38 | | | |
| | | | (0.17) | | | |
| | | | E41 | | | |
| | | | (0.17) | | | |
| | | | E44 | | | |
| | | | (0.42) | | | |
| 27 | ONT | 12 | E37 | 48.315 | -84.558 | NMC 24201- |
| | | | (0.31) | | | 1 – 24201-13 |
| | | | E38 | | | |
| | | | (0.31) | | | |
| | | | E40 | | | |
| | | | (0.31) | | | |
| | | | E42 | | | |
| | | | (0.08) | | | |
| 28 | NY | 12 | E26 | 42.989 | -76.771 | N/A |
| | | | (0.08) | | | |
| | | | W28 | | | |
| | | | (0.50) | | | |
| | | | E30 | | | |
| | | | (0.08) | | | |
| | | | E34 | | | |
| | | | (0.08) | | | |
| | | | E35 | | | |
| | | | (0.08) | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | E38 (0.17) | | | |
| 29 | ONT | 12 | E19 (0.83) E22 (0.08) E38 (0.08) | 45.0687 | -75.653 | N/A |
| 30 | MA | 12 | E19 (0.33) E24 (0.08) E25 (0.08) E26 (0.33) E28 (0.08) E32 (0.08) | 42.167 | -72.410 | NMC 23812-1,2,3; 23805-2; 23862-1 – 23862-4; 23869-1 – 23869-5 |
| 31 | QUE | 12 | E19 (0.33) E20 (0.25) E21 | 45.395 | -73.960 | NMC 17112-1 – 17112-12 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | (0.25) | | | |
| | | | E23 | | | |
| | | | (0.08) | | | |
| | | | E26 | | | |
| | | | (0.08) | | | |
| 32 | NB | 5 | E26 | 45.880 | -66.040 | NMC 24237- |
| | | | (0.40) | | | 3; 24302; |
| | | | E27 | | | 24319; |
| | | | (0.60) | | | 24320-1,2 |
| 33 | PEI | 12 | E26 | 46.361 | -62.529 | N/A |
| | | | (1.00) | | | |
| 34 | ONT | 12 | E28 | 51.278 | -80.635 | NMC 23818; |
| | | | (0.33) | | | 23893-1 – |
| | | | E38 | | | 23893-4; |
| | | | (0.67) | | | 23900; |
| | | | | | | 24221-2, |
| | | | | | | 24223-1 – |
| | | | | | | 24223-4 |
| 35 | QUE | 12 | E19 | 48.101 | -77.397 | NMC 24301- |
| | | | (0.67) | | | 1 – 24301-12 |
| | | | E28 | | | |
| | | | (0.33) | | | |

# CHAPTER 4.
# HISTORICAL DATA REFUTE RECENT RANGE CONTRACTION AS CAUSE OF LOW GENETIC DIVERSITY IN ISOLATED FROG POPULATIONS

ABSTRACT

Following the contraction of a species' range, isolated remnant populations may persist outside of the new continuous range. Such populations are often found to be less genetically diverse than populations in the interior of the species' range. One management strategy for rescuing isolated and genetically depauperate populations is artificial gene flow (Hedrick 1995, Land and Lacy 2000). Artificial gene flow has drawbacks, however, such as the possibility of introducing either diseases or genes that are maladapted to the local environment (Hedrick et al. 2000, Waples 1991). Another argument against artificial gene flow in such situations is that populations remaining after a range contraction were originally at the periphery of the species' old range. Evolutionarily important peripheral populations tend to naturally have lower diversity than interior populations owing to a history of isolation, founder effects, and chronically smaller population sizes. Therefore, one should be cautious about concluding that remnants have lost diversity and are in need of intervention. This study tested whether low genetic diversity in remnant populations of a declining amphibian is best explained by recent bottlenecks or by a history of being peripheral. I compared genetic diversity in historical and extant samples from populations in the interior and former periphery of the species' range. I found that historic peripheral populations already had reduced levels of genetic variation. Therefore, low diversity in the remnants could not be ascribed to the recent range contraction. This study shows that a common conservation strategy for rescuing genetically depauperate populations, artificial gene flow, may be unwarranted and even detrimental to evolutionarily important peripheral populations.

## INTRODUCTION

If a normally out-bred population is isolated and reduced in size, it can be put at increased risk of extinction owing to genetic drift and inbreeding depression (Keller and Waller 2002). One management strategy for rescuing isolated and genetically depauperate populations is artificial gene flow, the introduction of individuals from other populations (Hedrick 1995, Land and Lacy 2000). Because of recent data on the success of population supplementation (Madsen et al. 1999) as a counter-active measure for decreasing genetic variation and the costs therein, artificial gene flow is now a popular method for "rescuing" genetically depauperate populations. Artificial gene flow has drawbacks, however, such as the possibility of introducing diseases or genes that are maladapted to the local environment (Kedrick et al. 2000, Waples 1991). Thus, careful consideration of the causes of reduced variation in a population is necessary before supplementation is contemplated.

Contraction of geographic range is a common occurrence for many species in today's global environment (Channell and Lonolino 2000). During the contraction process, isolated ("remnant") populations may persist outside the edge of the newly contracted range. If these populations are found to be less genetically diverse than populations inside the species' contiguous range ("interior" populations), it is often assumed that the remnant populations have lost diversity. However, there is another potential explanation. Because ranges usually contract from the periphery (Brown 1995), it may be that remnant populations were originally on the periphery of the species' continuous range.

Peripheral populations tend to have lower diversity than interior populations owing to isolation, founder effects, and chronically smaller population sizes (Lessica and Allendorf 1995). Thus, if remnant populations were originally peripheral populations, then they may have always had lower genetic diversity than did interior populations. In these populations artificial gene flow may not be warranted. Although there have been a few studies that compared current vs. historical diversity in various populations (Bouzat et al. 1998, Matocq and Villablanca 2001, Pertoldi et al. 2001, Rosenbaum et al. 2000), there has never been a test of whether low diversity in a remnant population resulted from a recent range contraction or from a history of being peripheral.

Northern leopard frogs, *Rana pipiens*, have undergone a major range contraction in the Pacific Northwest since the 1980's (Leonard et al. 1999). However, recent surveys in the Pacific Northwest revealed a few isolated populations that persist outside of what is now the species' contiguous range (Fig. 4.1). Therefore, a comparison of genetic diversity in remnant (formerly peripheral) and interior populations with that found in historical populations taken from those same regions before the range contraction makes for a model system to test hypotheses about the causes of low genetic diversity in these remnant populations.

In this study I tested whether low genetic diversity in remnant populations of Pacific Northwest northern leopard frogs is best explained by a recent bottleneck or by a history of being peripheral. First, I assayed mitochondrial DNA (mtDNA) variation to verify that the putative remnant populations were actually native remnants, and not introduced populations from

laboratory stock or the pet trade. Second, I used eight microsatellite loci to compare genetic diversity in remnants with diversity in historical samples from the same region (i.e. the former periphery of the species range) that were collected before the population decline. I also compared genetic diversity in historical and extant interior populations. I conclude by discussing the implications of the results for management of the evolutionary legacy of *R. pipiens* and of peripheral populations in general.

## METHODS

Leopard frog samples were collected from 10 populations in the Pacific Northwest (Fig. 4.1). Populations were categorized according to location (peripheral or interior) and age of samples (extant or historic). "Remnant" populations were originally at the edge of the historic range of *R. pipiens*, but are now located outside what is currently the contiguous species range (Fig 4.1). "Interior" sites are still within the continuous range of the species. All extant samples consisted of toe clips collected during the summers of 1999 - 2000 and preserved by desiccation in 1.5 ml tubes filled with drie-rite desiccant. The historic population samples consisted of frozen liver in 95% ethanol from the Museum of Vertebrate Zoology, UC Berkeley (PARKCITY, MVZ#'s 501161 – 501170; collected in 1975) or dried skins (collected by F. W. Schueler) from the Canadian Museum of Nature (STEELE, CMN#'s 16081-1 – 16081-15; CYPRESS, CMN#'s 16089-1 – 16089-10; collected in 1971). Historic and extant population locality information is provided in Table 1. Total genomic

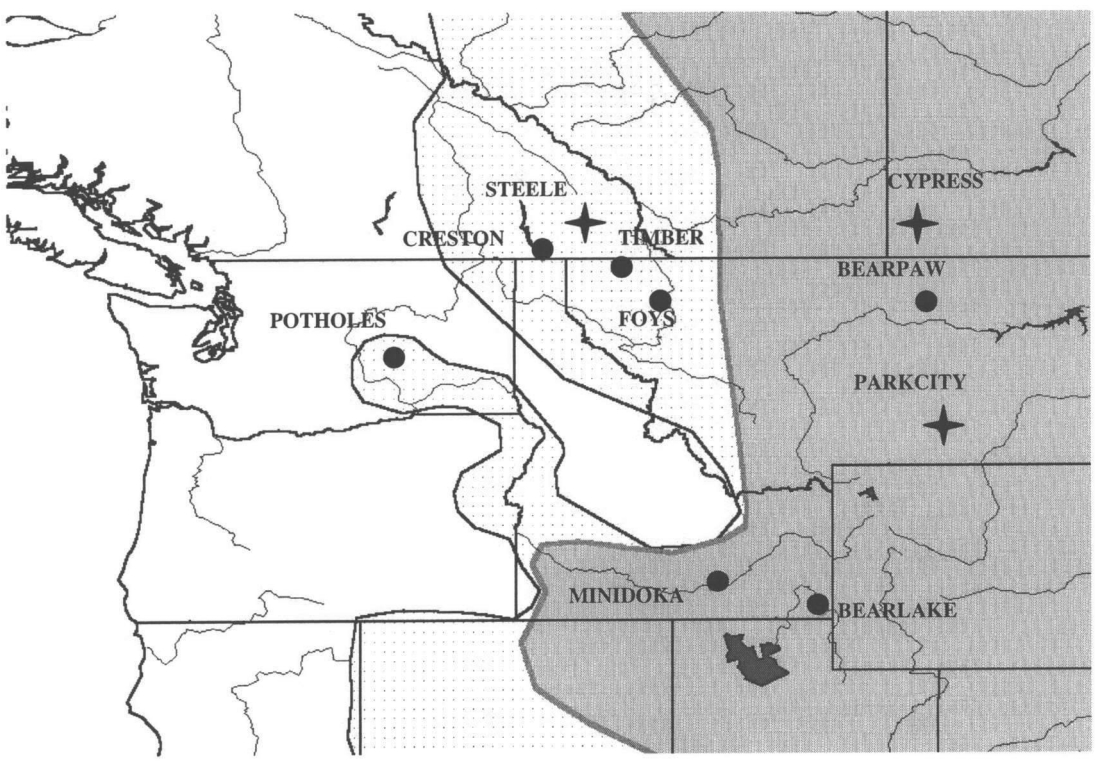
DNA was extracted following a standard phenol/chloroform technique (Samrook et al. 1999).

For mtDNA sequence analysis the 5' region of the mtDNA NADH dehydrogenase subunit 1 (ND1) gene, including the upstream tRNA –leucine were amplified by polymerase chain reaction (PCR) using primer MB77 (5'-TGGCAGAGCTTGGTTATGCAAAAGA-3') and primer MB145 (5'-CTAAGAGGAAGACACTCCAG-3') located in the central region of the ND1 gene. PCR reactions were carried out in 25 ul reactions using standard buffer conditions, 1.5 mM $MgCl_2$, 10 mM each dNTP, 0.5 Units *Taq* DNA polymerase and approximately 100 ng DNA. After 3 min. of initial denaturation, each of 35 cycles consisted of the following steps: 94°C for 45s, 47°C for 30s, and 72°C for 1 min, followed by a 7 min. final extension. PCR product was purified using the MoBio Ultraclean PCR cleanup kit (Salana Beach, CA) and sequenced by the Nevada Genomics Center (Reno, NV). A few initial samples were sequenced in both directions and used as reference sequences. The remaining samples were sequenced in only one direction with any ambiguous reads sequenced in the reverse direction for verification. All sequences were aligned with a type sequence and checked for correct base calls in SEQED (1.0.3, Applied Biosystems, Inc.). After proofreading, all sequences were aligned using ClustalW (Thompson et al. 1994) and converted to nexus format for data analysis. Sequence from 644 bp of mtDNA was obtained for 6 - 15 samples in each population. TCS (Clement et al. 2000) was used to create an intraspecific network of haplotypes (Figure 4.2) using the 95% statistical parsimony method of Templeton et al. (Templeton et al. 1992). For

confirmation of network topology, an intraspecific phylogenetic tree of unique haplotypes was constructed with PAUP* (4.0b, Swofford 2002) using maximum likelihood (ML) optimality criterion (using the HKY model of sequence evolution (Haseqawa et al. 1985) as determined by Modeltest v. 3.0 (Posada and Crandall 1998)). Parameter space was searched for the best tree employing a heuristic search conducted with 10 random additions and TBR branch swapping. In addition to the Pacific Northwest *R. pipiens* samples, a *R. pipiens* individual from the eastern part of the species range (Ohio) was also included in the phylogenetic analysis both as an outgroup and as a representative from the region where most commercial frogs are collected (Hoffman unpublished data).

Of the eight microsatellite markers used, six loci were developed for *R. pipiens* (Rpi100, Rpi101, Rpi103, Rpi104, Rpi107, and Rpi108) (Hoffman et al. 2003) and two loci were originally developed for *R. pretiosa* (RP193 and RP415; Blouin, unpub. data). PCR conditions were as follows: 3 min. of initial denaturation, followed by 35 cycles of the following steps: 94°C for 45s, annealing temp. for 30s, and 72° for 1 min, followed by a 7 min. final extension. Annealing temperatures and allele detection conditions are the same as described in Hoffman et al. (2003) The annealing temperatures of RP193 and RP415 were 44 °C and 51 °C respectively. Microsatellite data were analyzed with GENEPOP version 3.3 (Raymond and Rousset 1995) to test for Hardy-Weinberg equilibrium (exact tests, applying a sequential Bonferroni correction (Rice 1989)). *A priori* contrasts of within-population levels of genetic diversity were conducted between remnant versus extant interior

Figure 4.1. Map of the northwest edge of the species range of *Rana pipiens*. Historic western reaches of the species range (Stebbins 1995) and the new species range after a major contraction are both marked (Historic range – speckle, current range – shading). Population locations are marked with population name. Extant populations are designated by circles and historical samples are designated by stars.



populations, historical peripheral versus historical interior populations, and remnant versus historic peripheral populations. Differences in within-population levels of genetic diversity among sampling sites were assessed by one-way analysis of variance (ANOVA) using SYSTAT version 9.0 (for the PC, Systat Inc., Evanston, USA). Model parameters included either average expected heterozygosities or average number of alleles per locus as the main effect; inter-locus variation was accounted for as a blocking variable. To account for differences among populations in sample size, alleles per locus were estimated at the lowest common sample size via rarefaction (Hurlburt 1971) using POPULATIONS version 1.2.28 (© Langella 2000). Unbiased heterozygosites were calculated according to Nei (1987) by Fstat version 2.9.3 (© Goudet 2001). A sequential Bonferroni test (Rice 1989) was used to adjust significance levels for multiple comparisons.

## RESULTS

Statistical parsimony and ML networks exhibit identical topologies (Fig 4.2) and show a northwest group (haplotypes A, B, C) and a southwest group (haplotypes D, E, F) of Pacific Northwest haplotypes (Table 4.1). The sequence from Ohio was markedly different and provided evidence that no 'eastern' frogs were present in the populations sampled. Moreover, as would be expected for native frogs, no major population differences were detected between extant and historical populations. In fact, extant populations consisted of the haplotypes that would have been predicted given the closest historic populations.

Therefore, I am confident that the remnant populations are indeed native isolates.

Microsatellite data are summarized in Table 4.1. Only one locus in a single population showed a deviation from Hardy-Weinberg equilibrium (Creston at Rpi103), but all individuals amplified at this locus. Therefore, populations appear to be random mating, and there may be a low-frequency null allele at Rpi103 in Creston. Remnant populations had significantly lower average number of alleles per locus, $n_a$, ($F=33.4_{(1,47)}$, $p<0.001$) and lower expected heterozygosity, $H_e$, ($F=14.6_{(1,47)}$, $p<0.001$) than extant interior populations. Similarly, the historic peripheral population had significantly lower $n_a$ ($F=22.0_{(1,15)}$, $p=0.001$) and $H_e$ ($F=19.7_{(1,15)}$, $p=0.001$) than historic interior populations. Within each region, historic and extant populations did not differ ($n_a$: $F=0.016_{(1,31)}$, $p=0.90$ for remnant vs. periphery, and $F=1.69_{(1,31)}$, $p=0.20$ for extant vs. historic interior; $H_e$: $F=0.032_{(1,31)}$, $p=0.86$ for remnant vs. periphery, and $F=1.63_{(1,31)}$, $p=0.21$ for extant vs. historic interior). A plot of per population expected heterozygosity versus number of alleles per locus indicates a strong reduction in both variables in all remnant/peripheral populations (Fig. 4.3).

## Discussion

Mitochondrial sequence data indicate that peripheral populations of the northern leopard frog, *R. pipiens,* in the Pacific Northwest are not introduced from other localities and are not likely to be liberated laboratory frogs. They appear to be native, remnant populations. Support for this conclusion stems from three sources of evidence: First, there is genetic similarity among all

extant populations investigated from the Pacific Northwest. Second, there is genetic similarity among extant peripheral populations and historical populations from the same vicinity that were collected before the population contraction occurred. Third, there is marked genetic distinctness between populations in the Pacific Northwest and an eastern mtDNA haplotype that represents the region where most commercial frogs are collected. The comparison of genetic variation between historical edge versus historical interior populations revealed that peripheral populations were genetically depauperate before the range contraction occurred. Furthermore, Figure 4.3 shows that historical populations fall exactly within the range of genetic variation within extant samples from the same region. Clearly, peripheral populations of *R. pipiens* already had reduced levels of genetic variation. Therefore, low diversity in the remnants cannot be ascribed to the recent range contraction.

These results support the hypothesis that low diversity in remnant populations results simply because they were originally at the periphery of the species' range and not because of recently observed population declines. This study illustrates the importance of using historical information to make accurate management decisions. The usual comparison involving only extant populations could have misled managers into proposing an unwarranted augmentation of the remnant populations using frogs from the interior. Artificial gene flow into peripheral populations such as those in this study may be a particularly bad idea due to the potential costs associated with supplementation. Negative genetic effects of population supplementation can

include decreasing effective population size and reducing the genetic variation of the recipient population, as well as outbreeding depression (Tufto 2001). Recent studies investigating evolutionary models of the effects of gene flow from interior to peripheral populations have determined that supplementation can hinder adaptation (Kirkpatrick and Barton 1997) and increase the probability that peripheral populations will go extinct (Boulding and Hay 2001) by decreasing fitness in recipient populations.

In addition to the genetic disadvantages of artificial gene flow, there are additional ecological and evolutionary costs associated with supplementation of peripheral populations with animals from elsewhere in the species' range. The introduction of novel diseases or parasites and the potential for competition between natives and non-natives may have negative ecological effects. Evolutionarily, peripheral populations are important in spite of their naturally occurring reduced levels of genetic variation. Because peripheral populations are at the interface of species boundaries, they are essential for understanding the evolutionary processes involved in species boundary determination (Hoffmann and Blows 1994). Both empirical studies (Chapin and Chapin 1981) and genetic models (Garcia-Ramos and Kirkpatrick 1997) suggest that selection drives variation in species' fitness between interior and peripheral populations. Because of this adaptive potential, peripheral populations may be hotspots of evolution owing to their opportunity to drift and adapt to novel environments (Pertoldi et al. 2001, Garcia-Ramos and Kirkpatrick 1997). In sum, artificial gene flow and the subsequent loss of genetic distinctness of peripheral populations could do serious damage to the evolutionary legacy of a species.

Figure 4.2. Haplotype network for the mitochondrial DNA haplotypes of United States Pacific Northwest populations of the northern leopard frog. The network was created using both statistical parsimony and ML criterion. Both methods exhibited identical topologies. For locations of haplotypes see Table 1. Circles represent haplotypes, with size roughly estimating frequency found among all samples. Straight lines connecting haplotypes represent single base pair changes and dark rectangles represent inferred haplotypes. Dashed line connecting haplotype D with East represents 18 mutational steps. Confidence in this connection is below the limits of 95% statistical parsimony, but is linked according to maximum likelihood.

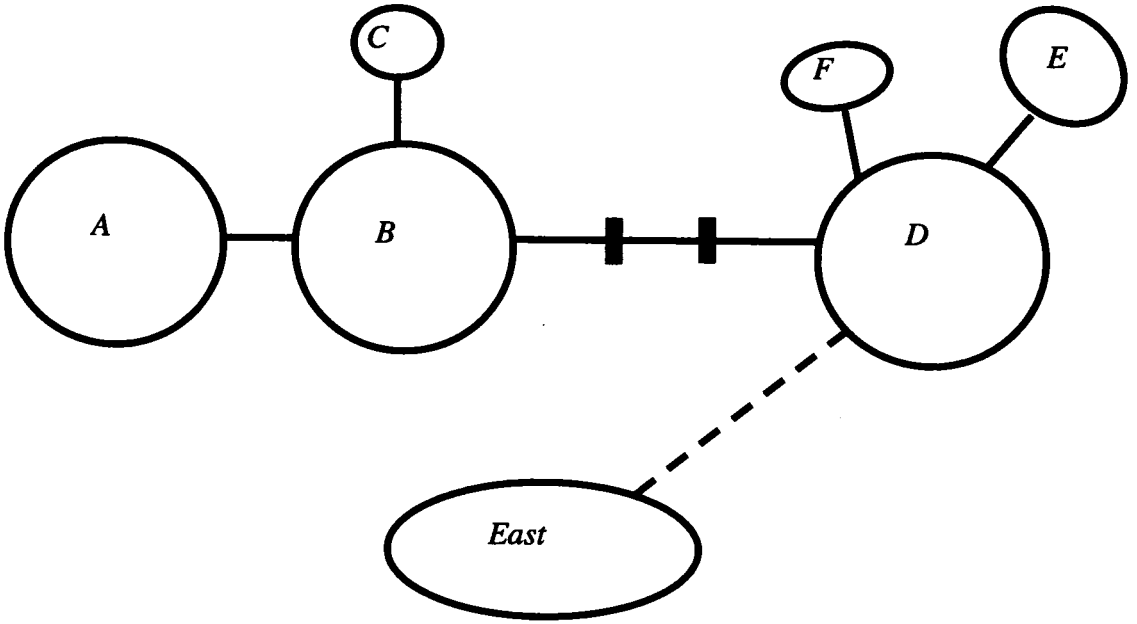


Table 4.1. Table of population name, location, category (E=Extant, H=Historic, P=peripheral, I=Interior, R=Remnant), mtDNA data, and microsatellite summary statistics.

| | | | | MtDNA Data | | Microsatellite Data | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | mean heterozygosity (+/-SE) | |
| location | latitude | longitude | category | sample size | haplotype | mean sample size (+/-SE) | mean alleles/locus (+/-SE) {rarified to sample size =5} | direct-count (+/-SE) | HWE expected (+/-SE) |
| Steele | 49.711 | -115.739 | H-P | 15 | A | 14.88 (.13) | 2.13 (0.40) {1.9 (0.28)} | .304 (.10) | .304 (.10) |
| Cypress | 49.658 | -109.494 | H-I | 10 | B,C | 10 (0) | 5.5 (0.87) {4.2 (0.56)} | .725 (.08) | .695 (.06) |
| ParkCity | 45.69283 | -108.984 | H-I | 9 | B,D | 9 (0) | 3.88 (0.77) {3.5 (0.61)} | .556 (.13) | .585 (.13) |
| Potholes | 47.07613 | -119.3536 | R | 12 | D,E | 29 (0) | 2.63 (0.50) {1.9 (0.26)} | .284 (.08) | .291 (.09) |
| Creston | 49.05 | -116.5017 | R | 12 | A | 26.25 (.37) | 2.63 (0.38) {2.3 (0.28)} | .413 (.09) | .435 (.08) |
| Timber | 48.814 | -115.001 | R | 6 | A | 5.50 (.27) | 2.0 (0.33) {2.0 (0.32)} | .392 (.15) | .320 (.11) |
| Foys | 48.178 | -114.366 | R | 6 | B | 4.38 (.26) | 1.75 (0.25) {1.8 (0.25)} | .331 (.14) | .246 (.09) |
| BearPaw | 48.1591 | -109.2003 | E-I | 6 | B | 4.38 (.46) | 3.0 (0.5) {3.0 (0.49)} | .615 (.12) | .547 (.11) |
| BearLake | 42.1385 | -111.262 | E-I | 12 | D,F | 21.75 (.16) | 6.88 (1.16) {4.0 (0.54)} | .602 (.10) | .618 (.11) |
| Minidoka | 42.6198 | -113.2837 | E-I | 12 | D | 29.88 (.48) | 5.25 (1.00) {3.4 (0.51)} | .501 (.10) | .550 (.11) |

One conservation dilemma facing species like *R. pipiens* is whether we should be concerned about the loss of peripheral populations even though the species as a whole is not in danger of extinction. Clearly, the evolutionary potential of *R. pipiens* lies not only within interior populations but also within edge and remnant populations found across the periphery of the species' range. Action should be taken to make sure that anthropogenic factors do not continue to cause range contractions in this species. However, population supplementation into Pacific Northwest remnant populations is not the best strategy to preserve the evolutionary legacy of the species. Artificial gene flow to save small, isolated populations should only be used as a last resort and only when truly warranted.

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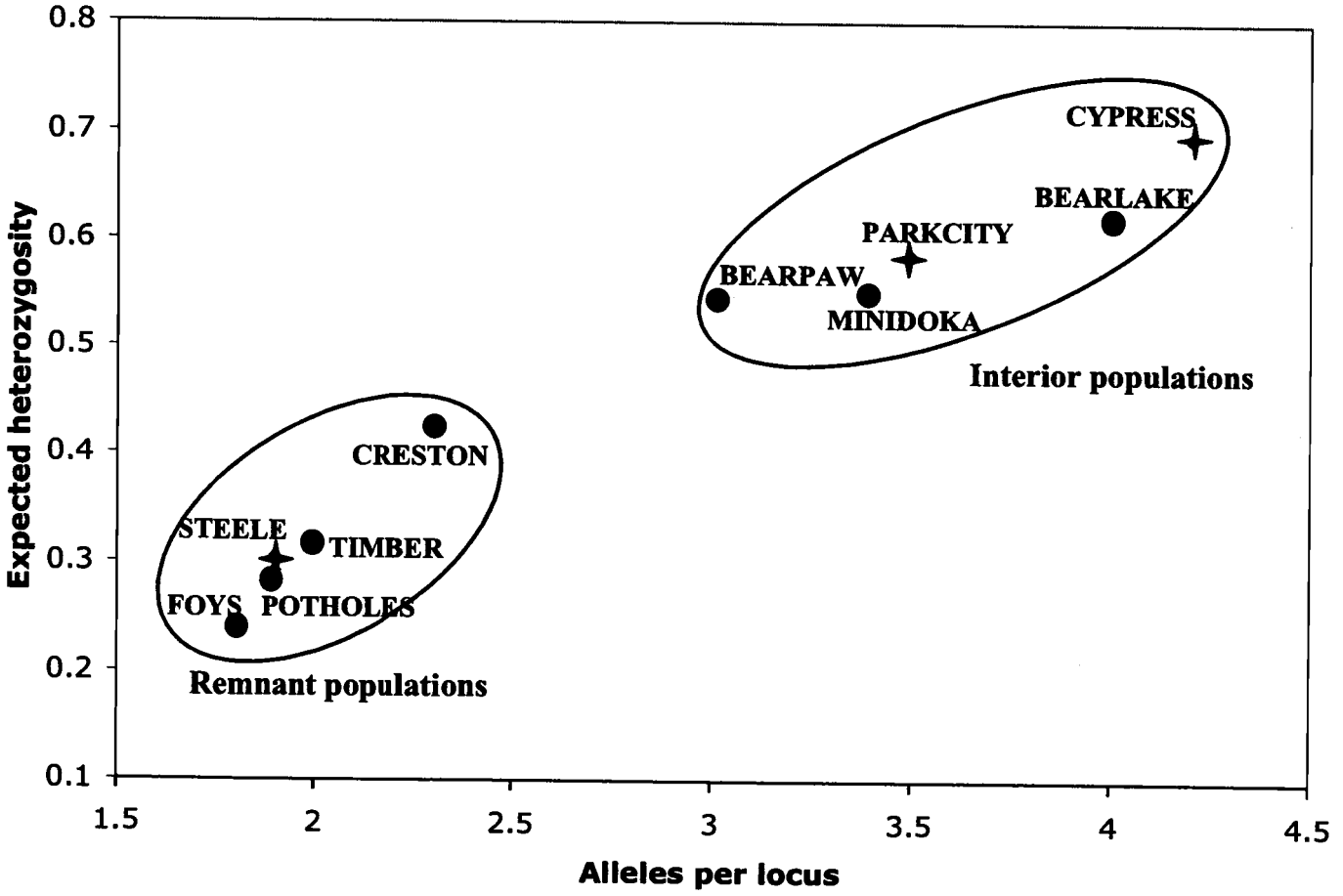
Figure 4.3. Plot of unbiased expected heterozygosity and number of alleles per locus adjusted to a common sample size of five via rarefaction (Hurlbert 1971). Both variables are strikingly reduced in all remnant/peripheral populations as compared to interior populations. Extant populations are designated by circles and historic samples are designated by stars.



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# CHAPTER 5.
# AN ANALYSIS OF SELECTION ON A COLOR POLYMORPHISM IN THE NORTHERN LEOPARD FROG

ABSTRACT

A primary focus of population genetics is to identify the role of microevolutionary processes in producing observed patterns of molecular and phenotypic variation. By determining genetic structure from a suite of putatively neutral molecular markers, one can determine if any loci exhibit patterns different than the neutral expectation. Therefore, genetic structure for any trait with a molecular basis can be compared to an expectation of neutrality. This technique (hereafter called the locus comparison approach) is a useful tool for testing whether the patterns of genetic variation within and among populations can be explained by genetic drift alone or if selection must be invoked. Color or pattern polymorphisms are excellent systems in which to evaluate whether a null hypothesis of genetic drift can be rejected in favor of an alternate hypothesis of selection to explain patterns of genetic variation. In this study, I tested whether a dorsal color polymorphism in natural populations of the northern leopard frog, *Rana pipiens*, is selectively maintained. Genetic structure at the color locus, assessed both spatially and temporally, was indistinguishable from structure at neutral loci. Moreover, simulations determined that although the strength of selection would need to be relatively strong to have been detected temporally, the spatial analysis would have detected all but very weak selection. This study exemplifies the importance of using the locus comparison approach to detect evidence for selective maintenance

before conducting studies to measure the selective mechanisms maintaining a polymorphism.

Introduction:

A primary focus of population genetics is to identify the role of microevolutionary forces in producing observed patterns of molecular and phenotypic variation. Genetic theory predicts that the frequency of polymorphic alleles is determined by the interaction among mutation rate, genetic drift, gene flow, and selection. However, most studies investigating the maintenance of a polymorphism with two or more high frequency alleles posit a selective mechanism without investigating non-selective (neutral) mechanisms for maintaining the polymorphism. In these studies the strength of selection on particular traits has traditionally been inferred from correlations between phenotype and components of fitness within populations (Endler 1986, Kingsolver et al. 2001). By necessity, these sorts of studies have usually been conducted within single populations. The extent to which phenotypic differences between populations can be explained by drift and migration compared to selection has received less attention.

One method to establish that a polymorphic trait is selectively maintained, as opposed to persisting as a neutral polymorphism governed by mutation and genetic drift, is to study multiple polymorphisms simultaneously. The availability of molecular markers readily provides numerous polymorphic characters enabling a general approach (hereafter called the locus comparison approach) for testing whether the level of genetic variation among populations can be explained by drift alone or if selection must be invoked. Because the demographic history of a species will influence all parts of the genome equally, genetic drift is expected to

leave a similar fingerprint across the entire genome. Under selection, however, only traits which are directly selected (and loci in linkage disequilibrium with these traits) exhibit the signature caused by the selective pressure. Therefore, selected loci should express a different genetic structure from the rest of the genome (Cavalli-Sforza 1966).

The locus comparison approach has primarily been applied to suites of molecular markers to determine post-hoc if any loci are putatively under selection. Bowcock et al. (1991) analyzed $F_{ST}$ plotted versus initial gene frequency for 100 human DNA polymorphisms and found an excess of $F_{ST}$ values with high and low values compared to expectations based on computer simulations of neutrally evolving loci. Beaumont and Nichols (1996) analyzed $F_{ST}$ plotted against heterozygosity for 117 polymorphic Drosophila loci and 27 polymorphic Atlantic cod loci. Beaumont and Nichols (1996) concluded that as many as eight *Drosophila* loci and at least two Atlantic cod loci were under selection as evidenced by $F_{ST}$ values at these loci greater than expected for neutral markers. Vitialis et al. (2001) analyzed a set of 43 polymorphic *Drosophila* loci and concluded that two are likely influenced by selection. Akey et al. (2002) analyzed 26,530 human single nucleotide polymorphisms (SNPs) and found 174 genes with a pattern of $F_{ST}$ that indicates they have been subject to natural selection.

The locus comparison approach has also been successful in combining molecular and phenotypic data to test the null hypothesis that genetic drift is sufficient to explain a particular pattern of phenotypic variation. Most of the

studies that have used the locus comparison approach to test for selection of phenotypic traits examined quantitative characters under selection (Prout and Barker 1993; Spitze 1993; Long and Singh 1995; Yang et al. 1996; Bonnin et al. 1996) or highly heritable traits thought to be under control of few loci (King and Lawson 1995; Mithen et al. 1995). Few researchers have used the locus comparison approach to examine particular alleles at individual loci under selection, and these studies typically involved loci under intense artificial selection from agents such as insecticides (Mithon et al. 1995; Chevillon et al. 1995; Taylor et al. 1995) rather than sources of more natural selection, such as predation.

Color or pattern polymorphisms are excellent systems in which to evaluate whether a null hypothesis of genetic drift can be rejected in favor of an alternate hypothesis of selection to explain patterns of genetic variation in natural populations. These polymorphisms often have simple Mendelian inheritance, and the phenotypes are easily scored (e.g. Gillespie and Oxford 1998; Subramaniam and Rausher 2000). Of the numerous studies attempting to measure selective mechanisms maintaining color polymorphism, only three recent studies have taken the locus comparison approach. Each of these studies (Gillespie and Oxford 1998; Andres et al. 2000, 2002) found that genetic variation among populations ($F_{ST}$) at the color locus was significantly less than variation among populations at putatively neutral loci.

In the present study, I test whether a dorsal color polymorphism in natural populations of the northern leopard frog, *Rana pipiens*, is selectively maintained.

*Rana pipiens* breeds during the spring, and tadpoles metamorphose from early summer to early fall. By metamorphosis, tadpoles exhibit a conspicuous polymorphism in which individuals develop either green or brown permanent coloration on their dorsum. This striking color polymorphism is controlled by a one locus, two-allele system in which the green allele is dominant to the brown allele (Fogleman et al. 1976). Thus, the easily scored phenotypic frequencies can be directly translated into genotypic frequencies, assuming populations are in Hardy-Weinberg Equilibrium (HWE), which can be assessed using co-dominant molecular markers. An understanding of the relationship between the genotype and the phenotype is one necessary criterion for investigating how natural selection can act on a phenotypic polymorphism.

*Rana pipiens* is an ideal species for investigating how selection acts on a polymorphism for several additional reasons. First, *R. pipiens* is widespread with a geographic range spanning much of North America (Conant and Collins 1991, see Fig. 1). Second, the polymorphism exists over the entire species range. These circumstances enable an investigation of how evolutionary forces act on the polymorphism under a variety of environmental conditions. Finally, past studies of mechanisms maintaining color polymorphisms in anurans provide a plausible hypothesis as to how the polymorphism could be selectively maintained. In a recent review of anuran color polymorphisms, Hoffman and Blouin (2000) compared previous studies looking at mechanisms maintaining polymorphisms in anurans and concluded that direct selection on the color polymorphism itself by

visually oriented predators seems the most likely explanation given anurans' position at the bottom of the vertebrate food chain. The field observations of snakes, herons, egrets, and birds of prey stalking adult and juvenile *R. pipiens* along with evidence of anti-predator behavior to some of these predators (Heinen and Hammond 1997), led me to conclude that direct selection on the color polymorphism by visually oriented predators is the most plausible mechanism maintaining the polymorphism in this species.

Previous experiments conducted with *R. pipiens* have suggested that the polymorphism is indeed selectively maintained. Schueler (1979) found a correlation between coloration and habitat and concluded that green frogs are favored in more forested habitats. Corn (1981) found a correlation between color and time of larval metamorphosis, such that brown frogs emerged from ponds earlier, when less green vegetation is present, than did green frogs. Moreover, studies have indicated that a different and unlinked spotting pattern polymorphism found in Minnesota populations of *R. pipiens* (burnsi morphs) may be selectively maintained (Merrell and Rodell 1968, Dapkus 1976).

In this study I aim to determine if there is a genetic signature of selection on the color locus in *R. pipiens*. Specifically, I take a locus comparison approach to identify different genetic patterns between putatively neutral loci (from molecular markers) and the color locus. First, I used both a large and small scale spatial approach to determine if the color locus exhibited a pattern of genetic variation among populations that is different from that at neutral loci. At a large spatial

scale, we might expect genetic variation among populations at the color locus to be less than that observed at neutral loci (smaller $F_{ST}$) owing to stabilizing selection on the color locus. Across a small spatial scale, we might expect either higher or lower $F_{ST}$ depending on whether the selectively maintained equilibrium allele frequencies are the same or different among populations. Second, I used temporally-spaced samples to determine if observed allele frequency change through time at the color locus is different than change through time at a simulated neutral bi-allelic locus. Finally, I used computer simulations to determine the relative values of the selection coefficient (assuming frequency-dependent selection or overdominance) necessary to be detected given my techniques. I then discuss the impact of my results for future studies testing the selective maintenance of polymorphism.

## METHODS

### *Samples*

Samples of *R. pipiens* consisted of two main classes, extant population collections and museum collections. Extant population tissue collections consisted entirely of toe clips collected during the summers of 1997 – 2001 and preserved by desiccation in 1.5 ml tubes filled with drie-rite desiccant. I recorded color and collected tissue from 22 – 48 randomly caught adult and sub-adult *R. pipiens* from 22 populations across the species range (Table 5.1, Figure 5.1). Museum

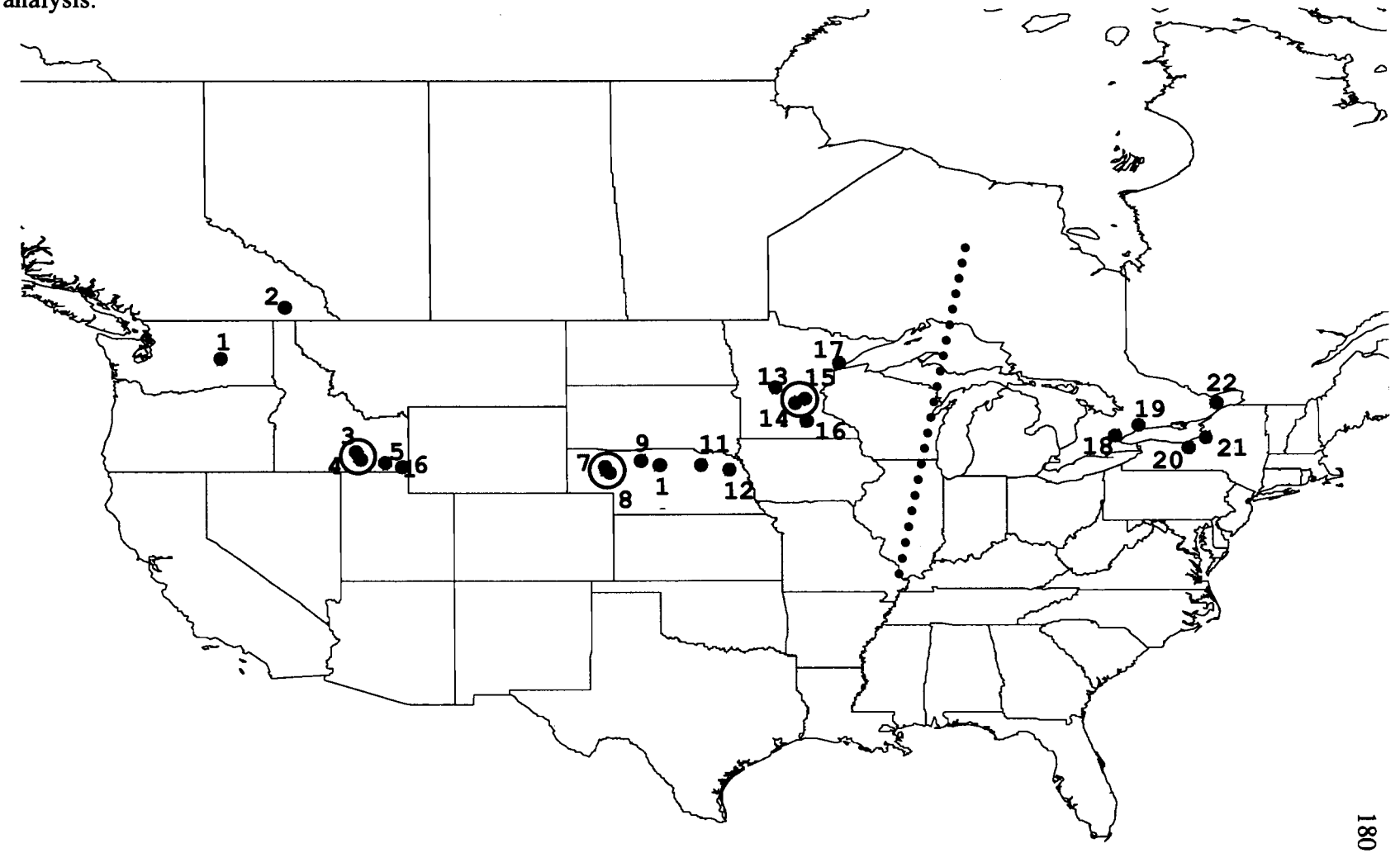
specimens from 5 populations consisted of samples originally collected in 1971 (Schueler 1979) that were preserved as dried skins (Schueler 1981), and catalogued at the Canadian Museum of Nature (Ottawa, Ontario). Color locus information from museum specimens was derived from field notes associated with each museum specimen. In all, I collected genetic and color locus information from 881 frogs in 22 populations (five of which had two collecting time periods; Figure 5.1) with an average of 33 frogs per population (Table 5.1). For analysis, collected samples were split into Eastern and Western regions owing to a large genetic split in the species' mtDNA phylogeny (Hoffman and Blouin, in review). By analyzing populations within regions, I decreased the probability of homoplasy in the genetic markers and thus increased the likelihood of accurately measuring genetic structure.

For molecular analysis total genomic DNA was extracted following a standard phenol/chloroform technique (Sambrook et al. 1989). Two molecular marker types were used to acquire neutral locus genetic structure. Seven microsatellite loci designed from *R. pipiens* (Rpi100, Rpi101, Rpi103, Rpi104, Rpi106, Rpi107, Rpi108) were used under the conditions described in Hoffman et al. (2003) and two microsatellite loci originally developed for *R. pretiosa* (RP193, RP415; Blouin, unpub. data) were used under the conditions in Hoffman and Blouin (in review). Microsatellite markers were tested for HWE using exact tests in Genepop version 3.3 (Raymond and Rousset 1995). Additional markers were

Table 5.1. Population identification (from Figure 1), state or province or population origin, analysis for which each population was used (L=large spatial area, S=small spatial area, T=temporal variation), number of samples per population, geographic coordinates of each population, and museum catalog number from the Canadian Museum of Nature for museum specimens.

| Population | Location | Analysis | N | Latitude | Longitude | Catalog Number |
|---|---|---|---|---|---|---|
| 1 | WA | L | 29 | 47.0761 | -119.3537 | |
| 2 | BC | L | 27 | 49.05 | -116.5017 | |
| 3 | ID | S, L | 23 | 42.6120 | -113.2473 | |
| 4 | ID | S, L | 31 | 42.6198 | -113.2837 | |
| 5 | ID | L | 23 | 42.2568 | -112.0132 | |
| 6 | ID | L | 22 | 42.4005 | -100.7893 | |
| 7 | NE | S, L | 31 | 41.9393 | -102.3872 | |
| 8 | NE | S, L | 22 | 41.9652 | -102.339 | |
| 9 | NE | L | 25 | 41.9467 | -102.4334 | |
| 10 | NE | L | 23 | 42.2371 | -100.0883 | |
| 11 | NE | L | 34 | 42.1734 | -98.1558 | |
| 12 | NE | L | 26 | 42.0006 | -97.00636 | |
| 13 | MN | L | 32 | 45.7097 | -94.89306 | |
| 14 | MN | S, L | 38 | 45.1264 | -94.0306 | |
| 15 | MN | S, L | 32 | 45.1917 | -93.6917 | |
| 16 | MN | L | 36 | 44.2153 | -93.5931 | |
| 17 | MN | L | 36 | 46.9208 | -92.1556 | |
| 18-1971 | ONT | T | 31 | 43.518 | -79.997 | 23890-1 – 29, 22687-1,2 |
| 18-2001 | ONT | L, T | 42 | | | |
| 19-1971 | ONT | T | 44 | 44.037 | -78.979 | 2456-1 – 22, 24257-1 – 22 |
| 19-2001 | ONT | L, T | 40 | | | |
| 20-1971 | NY | T | 42 | 42.9893 | -76.7715 | 23876-1 – 32, 23877-1 – 10 |
| 20-2001 | NY | L, T | 48 | | | |
| 21-1971 | NY | T | 53 | 43.4679 | -76.01 | 23842-1 – 15, 23851-1 – 22, 23856-1 – 17 |
| 21-2001 | NY | L, T | 22 | | | |
| 22-1979 | ONT | T | 31 | 45.0687 | -75.653 | 24332-1 – 39 |
| 22-2001 | ONT | L, T | 38 | | | |

Figure 5.1. Map of sampling localities. Dotted line indicates the East/West regional split. Circles around pairs of populations indicate populations used for small scale geographic range. All eastern populations were used for temporal as well as spatial analysis.



developed using the Inter Simple Sequence Repeat (ISSR) technique. This technique uses random primers anchored in microsatellite regions of DNA and produces large numbers of dominant loci that can be scored reliably (Zietkiewicz 1994). ISSR loci were optimized such that each PCR reaction was carried out in a 25 ul reaction under the following conditions, 3mM $MgCl_2$, 10 mM each dNTP, 1X PCR enhancer (MasterAmp$^{tm}$ Enhancer, Epicentre, Madison, WI), 0.2 uM primer 812 (UCB primer stock), 0.5 Units Taq DNA polymerase, and 20 ng DNA. The temperature profile consisted of an initial 3 min. denaturation step (94°C), then 40 cycles of 94°C for 30 sec., 46°C for 45 sec., and 72°C for 2 min., followed by a final extension (72°) for 7 min. All PCR reactions were carried out in a Perkin Elmer 9600. DNA concentrations were quantified with a Hoefer DyNAQuant 200 fluorometer (Hoefer Scientific Instruments, San Francisco, CA) and diluted to 100 ng/ul for each microsatellite PCR reaction and 10 ng/ul for ISSR PCR reactions. Both microsatellite and ISSR loci were scored with an internal lane standard on an ABI 3100 to increase reliability of scored markers. Data were scored using Genotyper 3.2 (Applied Biosystems).

*Spatial Analyses*

Spatial analysis was carried out using the procedure of Beaumont and Nichols (1996) as implemented by the computer program FDIST2 (located at http://www.rubic.rdg.ac.uk/~mab/software.html). Briefly, this program calculates $F_{ST}$ and heterozygosity according to the methods described by Cockerham and Weir

(1993). Then, the expected $F_{ST}$ is calculated from the data as the average among loci weighted by their heterozygosity. To build the null model in this study the data included all the microsatellite and ISSR loci. Coalescent simulations were then performed using samples of the same size and number as the data (i.e. number of populations in the analysis and median sample size of all populations), assuming an island model (with 100 islands) and an infinite alleles mutational model. $F_{ST}$ and heterozygosity were calculated, and this whole procedure was repeated 40,000 times. The confidence limits for the distribution of $F_{ST}$ as a function of heterozygosity was characterized by estimating where the least extreme 95% of the simulated data points were expected to lie (Beaumont and Nichols 1996). By plotting the observed values for the loci used in the genetic analysis, I determined whether any loci were outside of these 95% confidence limits.

Using this technique I conducted two different analyses. First, I tested for variation across broad geographic ranges. This large scale analysis included all populations in the west region or the east region, respectively (see Fig. 5.1). Second, I tested for variation across three small geographic ranges (mean distance between each population pair = 12 km; Fig. 5.1). By analyzing populations across this broad scope of distances I hoped to increase the likelihood of finding evidence for selection. In all of these analyses, the color locus was removed when simulating the expected distribution.

*Temporal Analyses*

The goal of the temporal analysis was to determine whether the allele frequency at the color locus had changed more or less over time than could be explained by genetic drift alone (see Fig. 5.1). To estimate the amount of genetic drift expected at the color locus in each population I first estimated the effective population size ($N_e$), the starting allele frequency of the color locus, and the number of generations that had ensued between sampling periods in the populations sampled. The starting allele frequency of the color locus was calculated from the museum data (see Table 5.2). The number of generations between sampling periods was calculated assuming two years per generation (Ryan 1953), and $N_e$ was estimated from temporal change at the microsatellite loci following the pseudo-maximum likelihood method of Wang (2001). This method performs equally well as a full likelihood method, but is less computationally intensive and is therefore ideal for highly polymorphic markers such as those in this study, in which I observed as many as 58 alleles per locus (Wang 2001). Although a recent method has been proposed for the joint measure of migration rate (m) and $N_e$ (Whitlock and Wang 2003), this technique was not used because I did not have an appropriate source population. Using the non-focal populations as sources produced unbelievable parameter estimates given data on cenus sizes, philopatry and home range size of leopard frogs (Dole 1965, Dole 1971, pers. observation). In addition, these estimates varied wildly depending on which populations were chosen as sources.

Table 5.2. Table of parameters used in simulation of temporal change in allele frequency. $P_0$ is the observed allele frequency at the color at generation 0. Ne is the effective population size as determined from the program MNE (Wang 2001). Generations denotes the number of generations of simulated allele frequency change. P′ is the observed allele frequency at the color locus after n (n=15 or 11) number of generations. ΔP is the observed change in allele frequency at the color locus. The observed variance in ΔP (see Figure 5) is 0.004.

| Population | $P_0$ | Ne | Generations | P′ | ΔP |
|---|---|---|---|---|---|
| Montezuma | 0.36 | 551 | 15 | 0.36 | 0 |
| Happy Valley | 0.49 | 102 | 15 | 0.54 | 0.05 |
| Fairmile | 0.68 | 243 | 11 | 0.64 | -0.04 |
| Nonquon | 0.62 | 324 | 15 | 0.72 | 0.1 |
| Campbellville | 0.58 | 205 | 15 | 0.53 | -0.05 |

For each population, I used estimated $N_e$, the starting allele frequency of the color locus, and the number of generations between sampling periods in a Monte Carlo simulation of genetic drift for a bi-allelic locus. Each generation, a uniformly distributed random number between zero and one is drawn. If the number lies between zero and the frequency of allele A in the parents, then gamete A is produced, whereas if the number lies between the frequency of A and one, then gamete B is produced. This calculation is done until the total number of gametes equals $2N_e$. The new frequency of allele A (after one generation of drift) is calculated from this pool of chosen gametes. The process is repeated for multiple generations to simulate drift over any desired span of time (Hedrick 2000).

To determine whether the change at the color locus from the five observed populations deviated significantly from expectations under drift, I simulated the simultaneous evolution of five populations with parameters equal to the observed populations (for model parameters see Table 5.2). From this I calculated the change in the allele frequency ($\Delta p$) for each population and the variance in $\Delta p$ ($\Delta p_{var}$) among all five simulated populations. The simulation was repeated 10,000 times to generate a distribution of $\Delta p_{var}$ under the hypothesis of neutrality. The $\Delta p_{var}$ of the observed populations was then compared to the distribution of simulated $\Delta p_{var}$. An observed value within the 2.5% tail at the low end of the distribution would indicate significantly low values of $\Delta p_{var}$, likely owing to stabilizing selection or unidirectional selection (in the same direction) for all five

populations. An observed value within the 2.5% tail at the high end of the distribution would indicate significantly high values of $\Delta p_{var}$ likely owing to distinct selection pressures in different populations. An observed value falling within the 95% body of the distribution would indicate that $\Delta p_{var}$ at the color locus could not be differentiated from a null hypothesis of variation as expected from genetic drift.

*Simulations to determine detectable selection coefficients*

*Spatial simulations*

To determine the power to detect selection over a broad spatial scale, I simulated the effects of selection on $F_{ST}$ in an array of evolving populations. I focused on the effects of two likely mechanisms of balancing selection: negative frequency-dependent selection and overdominance (Hedrick 2000, Allen 1988). The guiding principle behind these analyses is that balancing selection will tend to promote convergence in allele frequencies among populations. If the observed $F_{ST}$ is much greater than the $F_{ST}$ expected under a particular strength of balancing selection, then I can rule out that such selection is at work in the populations under consideration. General model parameters were roughly matched to the observed data. The number of simulated populations was 17 (the same as in the large scale spatial analysis). Effective population size was set to 284 per population (the mean $N_e$ estimated from the temporal analysis), and the starting allele frequency was set

to 0.5. I varied the values of selection coefficients to simulate a wide array of selection strengths.

These models included selection and drift. The basic drift model described above was modified to include a change in allele frequencies due to selection before the random sampling of gametes each generation. For frequency-dependent selection the equation for the single-generation change in allele frequency (from Wright 1984) is:

$$\Delta q = q^2(1-q)[(s_1 + s_2)q^2 - s_1]/\overline{W},$$

where

$$\overline{W} = (1 + s_1) - 2s_1q^2 + (s_1 + s_2)q^4.$$

For overdominance, the equation for the change in allele frequency over time (from Futuyma 1986) is:

$$\Delta q = [pq(s_1p - s_2q)]/(1 - s_1p^2 - s_2q^2).$$

The simulation allowed allele frequencies to evolve according to selection and drift for a random number of generations (between 500 and 700). In this type of simulation, with no migration or mutation, all populations will eventually go to fixation, so I chose a number of generations that allowed them to achieve quasi-equilibrium due to selection and drift. Seventeen allele frequencies (representing

the observed 17 populations) were obtained at the end of the simulation, and $F_{ST}$ was calculated for that set of populations. In all cases, I assumed that $s_1$ was equal to $s_2$. For each value of interest for $s_1$ and $s_2$ (see Table 5.3), the simulation was run 200 times to obtain estimates of mean $F_{ST}$ for a given selection coefficient.

*Temporal simulations*

To determine the strength of selection necessary to cause detectable temporal variation between neutral loci and the color locus, I again simulated the effects of frequency-dependent selection and overdominance. General model parameters were identical to those used to estimate $\Delta p_{var}$ in the original temporal analysis simulation. Number of populations simulated was five, $N_e$ was set to the estimate in each population from the temporal analysis, and the equilibrium allele frequency was set to the observed starting allele frequency in each population. For frequency-dependent selection and overdominance the equation for the change in allele frequency over time ($\Delta q$) was the same as in the spatial simulations. The value of the $s_1$ was set to a value of interest (ranging from -0.01 – -0.50 for frequency-dependent selection and 0.01 – 0.05 for overdominance), and the value of $s_2$ was calculated assuming that each population was initially at equilibrium. Thus, each population was allowed to have a different equilibrium and different values of $s_2$, while $s_1$ was held constant across populations as a general indicator of the strength of selection. The equilibrium gene frequency for frequency-dependent selection (from Wright 1984) is:

$$\hat{q} = \sqrt{s_1/(s_1 + s_2)},$$

so

$$s_2 = (s_1 - \hat{q}^2 s_1)/\hat{q}^2,$$

whereas for overdominance (from Futuyma 1986):

$$\hat{q} = s_1/(s_1 + s_2),$$

so

$$s_2 = [s_1(1 - \hat{q})]/\hat{q}.$$

The simulation allowed each population's allele frequencies to evolve according to drift and selection as in the spatial simulations of selection for the number of generations observed (11 or 15). At the end of the simulated number of generations $\Delta p$ was measured in each population and $\Delta p_{var}$ was calculated for all populations. The entire simulation was repeated 10,000 times, and the upper and lower 95% confidence limits were calculated for each value of the selection coefficient, as in the temporal simulations of drift described above.

Table 5.3. Coefficient of selection ($S_1$) and upper and lower 95% confidence intervals for $\_p_{var}$ calculated from two models of selection: frequency-dependent selection and overdominance. Simulation parameters were identical to those used in Table ?, except the selection coefficient was included in the model (see text). The point estimate of $\_p_{var}$ for the color locus was 0.004

| Model of Selection | Selection Coefficient | Lower 95% C.I. | Upper 95% C. I. |
|---|---|---|---|
| Frequency-Dependence | -0.05 | 0.000623 | 0.018319 |
| | -0.1 | 0.000477 | 0.014099 |
| | -0.2 | 0.000289 | 0.008804 |
| | -0.3 | 0.000177 | 0.005732 |
| | -0.4 | 0.000131 | 0.003999 |
| | -0.5 | 0.000098 | 0.002887 |
| Overdominance | 0.05 | 0.000583 | 0.017753 |
| | 0.1 | 0.000417 | 0.013518 |
| | 0.2 | 0.000268 | 0.008066 |
| | 0.3 | 0.000175 | 0.005327 |
| | 0.4 | 0.000126 | 0.003909 |
| | 0.5 | 0.000103 | 0.003112 |

## RESULTS

Because microsatellite markers showed that each population was in HWE (see below) the allele frequencies at the color locus were simply calculated in each population by taking the square root of the frequency of the brown frogs (homozygous recessive state) as the frequency of the brown allele. The frequency of the green allele was calculated as one minus the frequency of the brown allele.

### *Spatial analysis*

The microsatellite dataset consisted of two populations with loci that significantly deviated from HWE after a sequential Bonferroni correction (Rice 1989). In population 20 two loci deviated from HWE and in population 2 one locus deviated from HWE. Because of the rarity of loci out of HWE all loci for all populations were included in the FDIST2 analyses. Nineteen ISSR loci showed strong and repeatable electropherograms with Genotyper 3.2 and were scored for use in the FDIST2 analyses. As dominant markers, the ISSR loci were scored such that the presence of the band signified either the homozygous dominant or heterozygous state, and the absence of the band indicates the homozygous recessive state. Moreover, HWE could not be directly assessed, but was assumed given the population data from the microsatellites. Not all ISSR loci were polymorphic in all populations. Therefore, when an analyses was conducted between populations

where an ISSR locus was not polymorphic that locus was dropped, changing the total number of loci used among analyses.

In the western region, expected $F_{ST}$ at neutral loci was calculated from the data as 0.1888 for 17 populations (with 26 loci) and a median samples size of 29. Figure 5.2 shows the 95 percent confidence limits for the relationship between $F_{ST}$ and heterozygosity for simulated neutral loci. The point estimate of $F_{ST}$ as a function of heterozygosity at the color locus ($F_{ST} = 0.1973$) falls directly in the middle of the expected distribution. Similarly, in the eastern region, the point estimate of $F_{ST}$ as a function of heterozygosity at the color locus ($F_{ST} = 0.1398$) also falls directly in the middle of the expected distribution (Figure 5.2). Expected $F_{ST}$ for the neutral markers was calculated from the data as 0.071559 for the five eastern populations (with 25 loci) and a median sample size of 40. In both the east and west regions some putatively neutral markers used to create the expected distribution fall outside the 95% expected range. The majority of these markers are microsatellites and likely fall outside the expected distribution owing to their large numbers of alleles and hence their high heterozygosities. The values observed for one locus in the eastern distribution cannot be explained in this way; however, this locus likely falls outside the expected distribution due to chance (we would expect 1-2 loci to fall outside the 95 percent confidence limits by chance alone).

For the pair-wise small scale analysis, expected $F_{ST}$ at neutral loci was calculated as 0.016259 (with 19 loci), 0.12857 (with 24 loci), and 0.050198 (with 15 loci) respectively for the population pairs in Nebraska, Minnesota, and Idaho.

In each of these comparisons, two populations were analyzed with median sample sizes of 26 (Nebraska), 35 (Minnesota), and 26 (Idaho). In each comparison plot of $F_{ST}$ as a function of heterozygosity, the color locus again falls in the middle of the expected distribution (Figure 5.3; mean $F_{ST}$ of color locus: Nebraska $F_{ST}$=0.0405; Minnesota $F_{ST}$=0.0922; Idaho $F_{ST}$=-0.0105).

*Temporal Analysis*

Only seven microsatellite loci and none of the ISSR loci amplified consistently with the historic DNA samples (microsatellites not amplifying were Rpi104 and Rpi107). Therefore only these seven microsatellite loci were used in the temporal analysis. Maximum likelihood estimates of $N_e$ for each population are: population 18=205; population 19=324; population 20=551; population 21=102; population 22=243. Maximum likelihood surfaces for each analysis are plotted in Figure 5.4. Figure 5.5 displays the distribution of $\Delta p_{var}$, when these numbers were used to simulate genetic drift at a bi-allelic locus for each of five populations (with 95% of values falling between 0.001 and 0.0205).

Figure 5.2. Fdist2 results of $F_{ST}$ for simulated neutral markers as a function of heterozygosity over large scale spatial variation. The upper and lower lines indicate 0.975 and 0.025 quantiles, the middle line indicates the mean $F_{ST}$. Points on the figure indicate values of $F_{ST}$ and heterozygosity for molecular markers used to create the distribution. The star indicates the point estimate for the color locus.

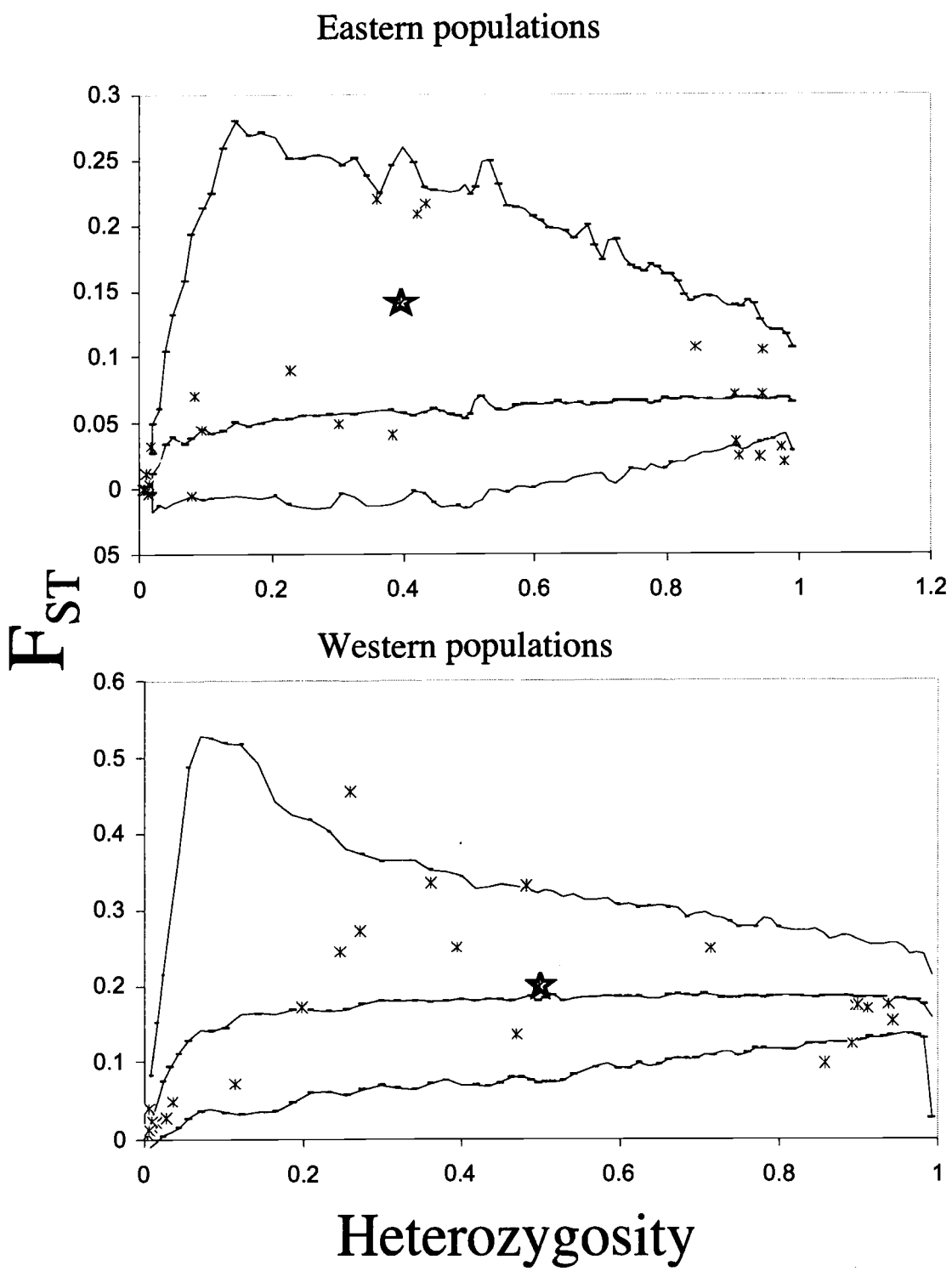


Figure 5.3. Fdist2 results of $F_{ST}$ as a function of heterozygosity over small scale spatial variation. The upper and lower lines indicate 0.975 and 0.025 quantiles, the middle line indicates the mean $F_{ST}$. Points on the figure indicate values of $F_{ST}$ and heterozygosity for molecular markers used to create the distribution. The star indicates the point estimate for the color locus.

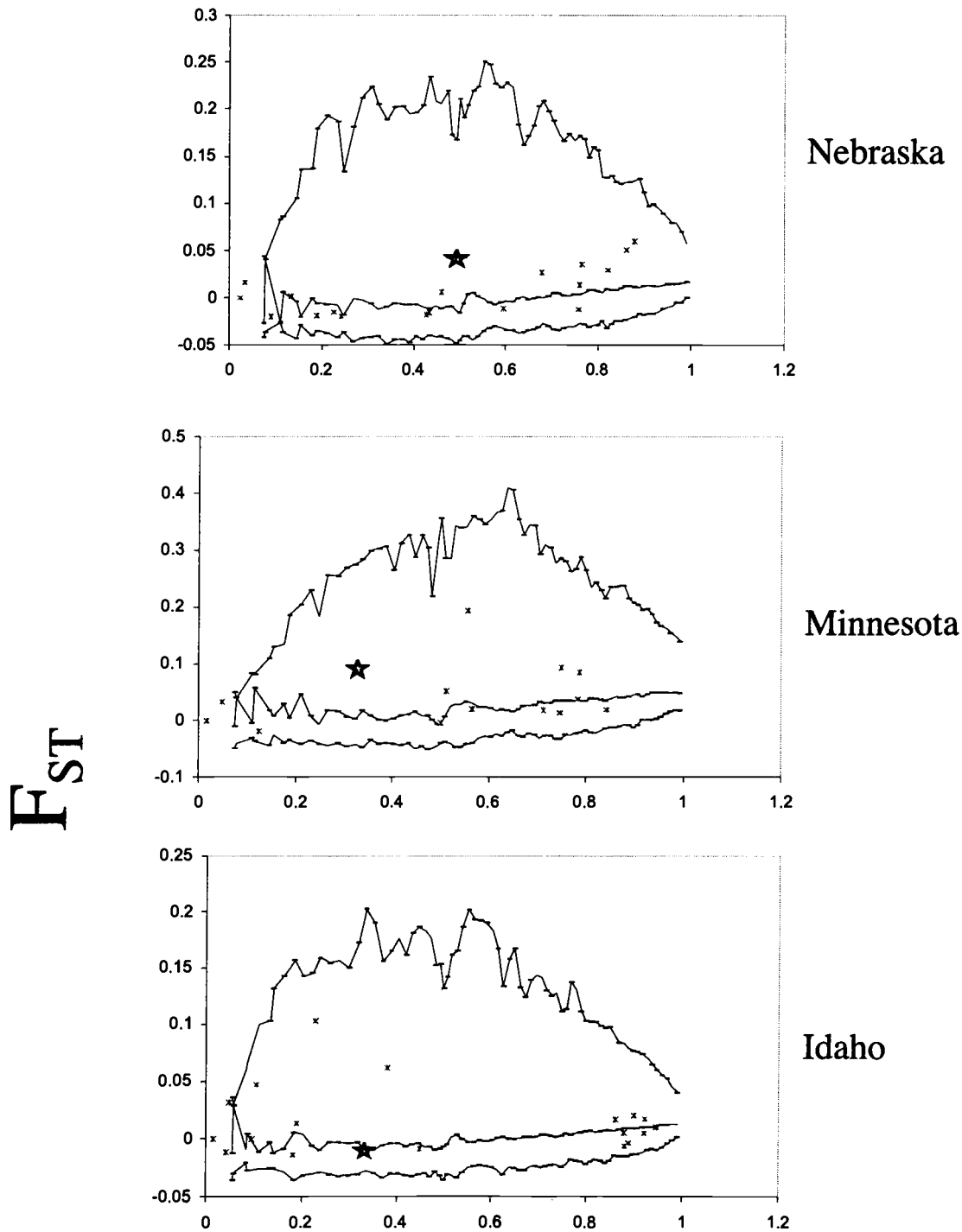


Figure 5.4. Likelihood surfaces for estimates of effective population size for each population used for temporal analysis of allele frequency change.

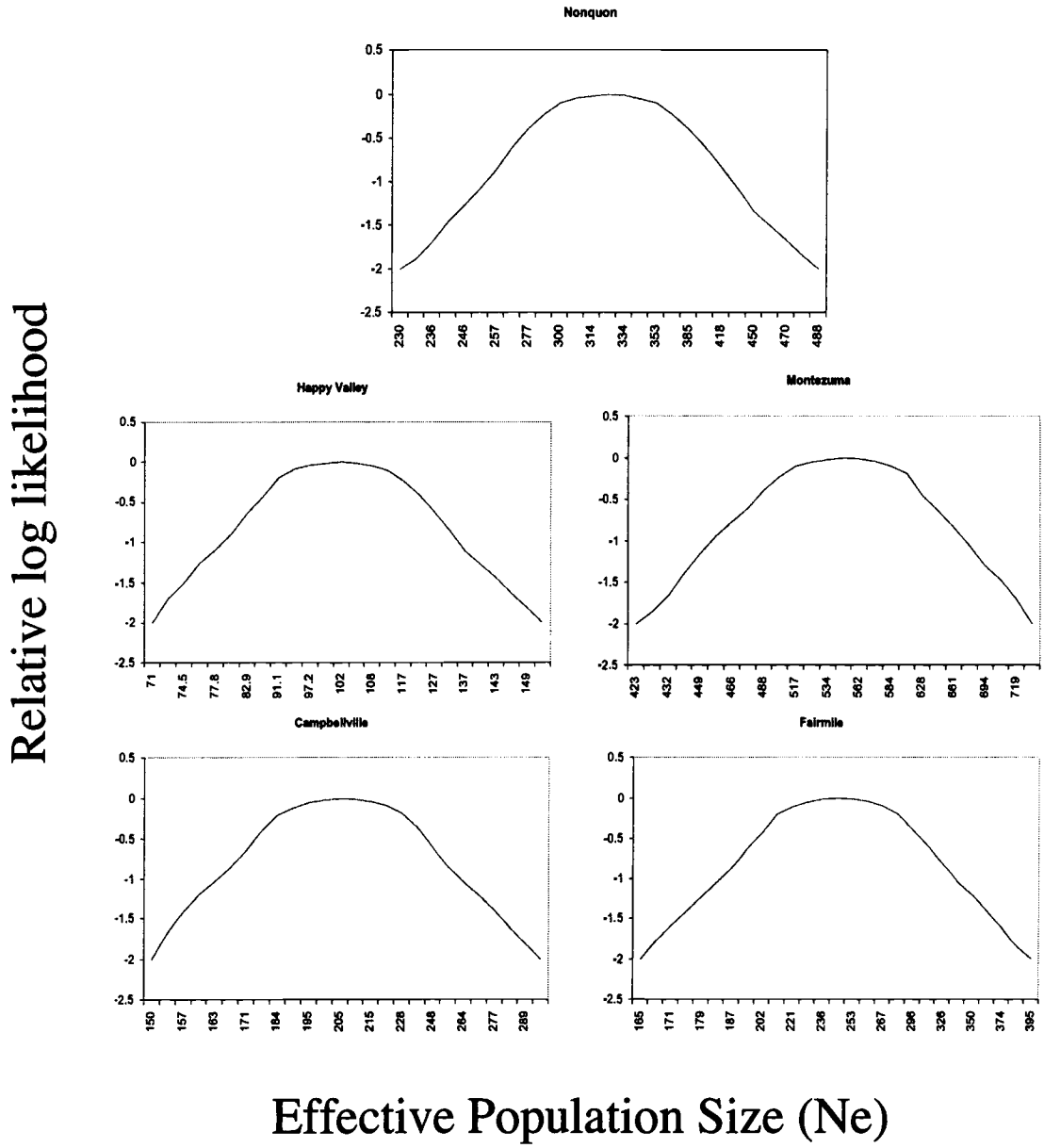
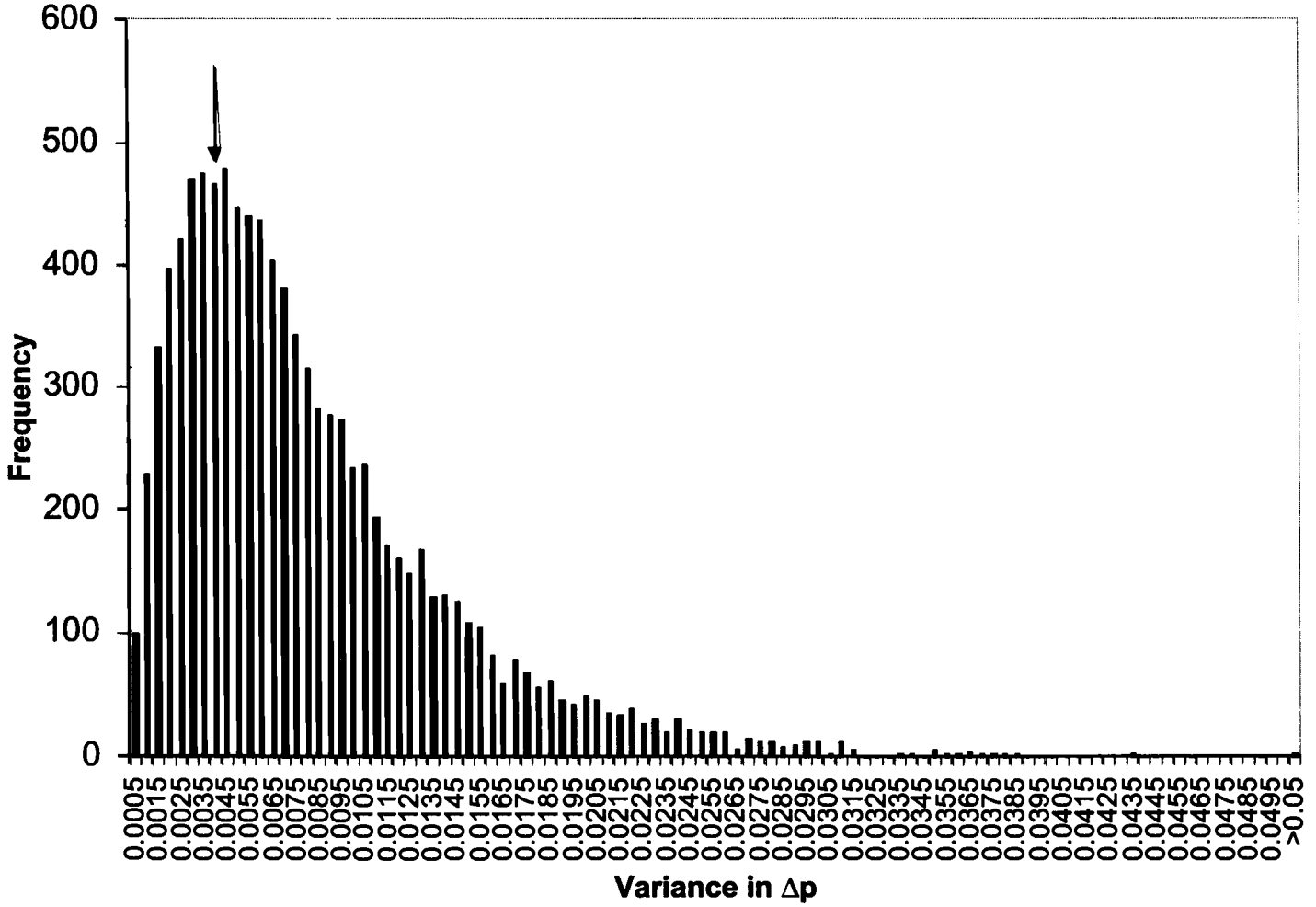


Figure 5.5. Graph of variance of Δp due to genetic drift for five populations simulated 10,000 times with parameters similar to those of the observed populations. Arrow indicates the value of the point estimate for the variance in Δp among the 5 observed populations. Darkened tails of graph indicate values outside 95% of simulated values.



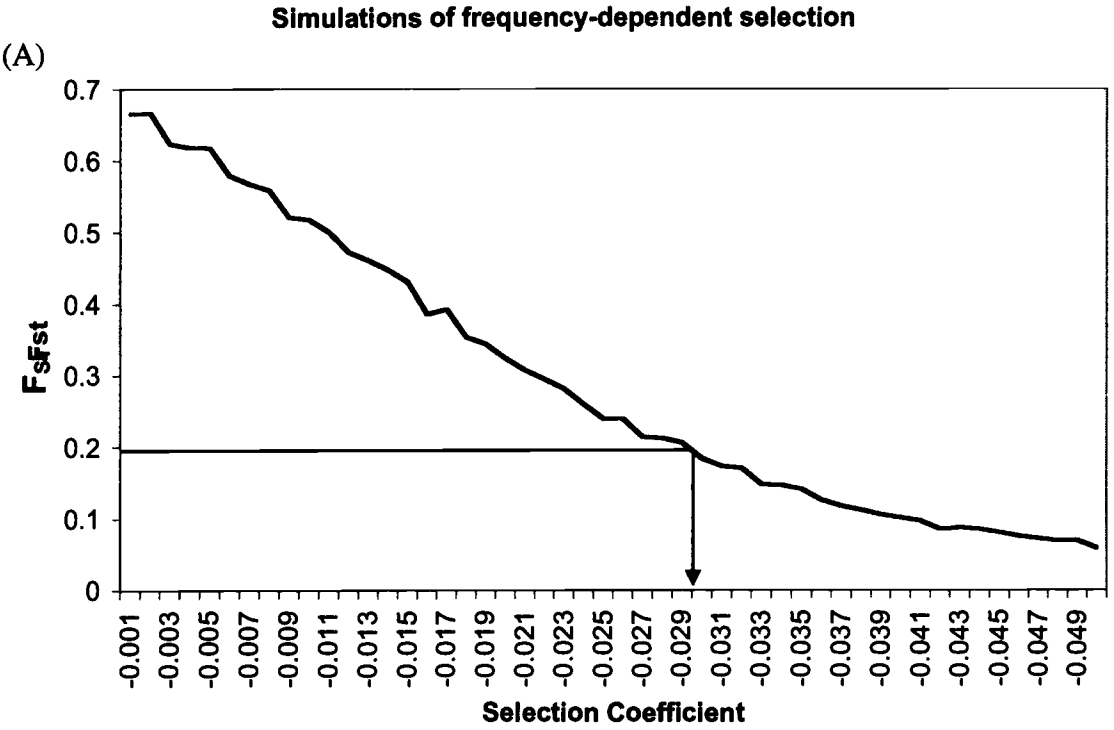
The $\Delta p_{var}$ from the five observed populations for the color locus was 0.004. Once again, the observed value of change at the color locus falls exactly in the middle of the distribution expected from putatively neutral loci.

*Simulations to determine detectable selection coefficients*

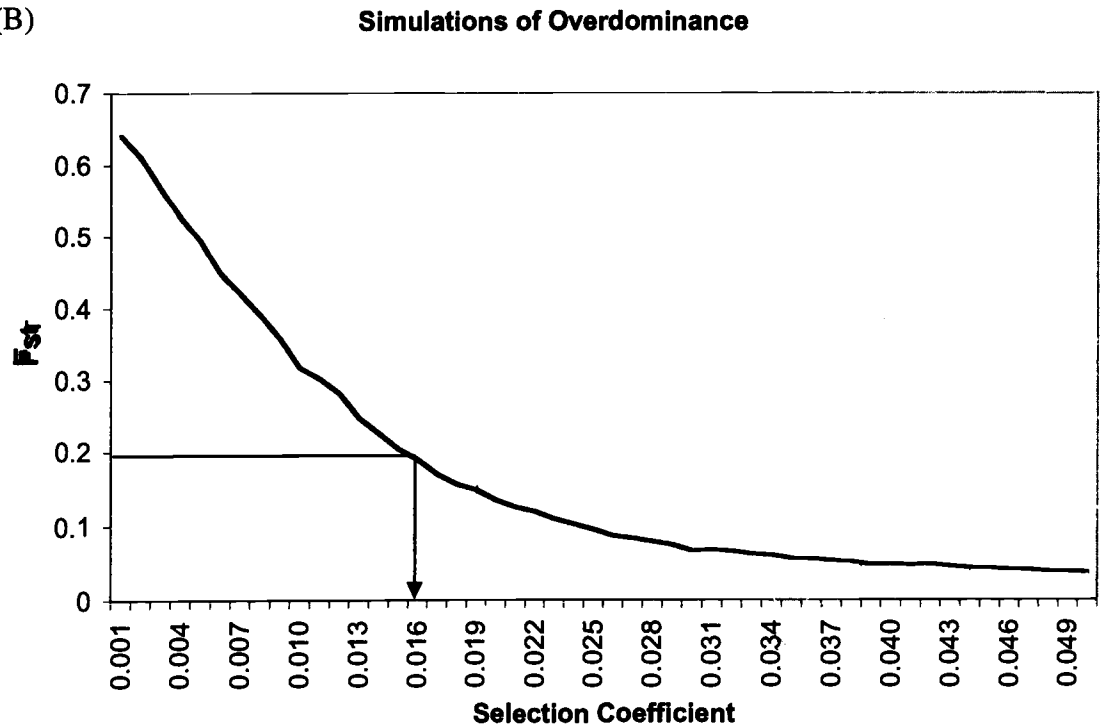
Spatial simulations indicated that the global $F_{ST}$ responds as expected to increasing values of selection coefficients (Figure 5.6). The simulations indicated that if the strength of selection was much stronger than would be produced by selection coefficients (i.e., $s_1$ and $s_2$) of about –0.029 under frequency-dependent selection or 0.016 under overdominance, then simulated population differentiation ($F_{ST}$) would be much less than was observed at the color locus. Therefore, if any selection is acting on the color polymorphism, the intensity of selection must be less than selection coefficients of approximately -0.029 (or 0.016) would produce. The simulation-based analysis of the temporal data suggests that I would have less power to detect selection from these samples. These simulations indicated that coefficients of selection greater than -0.4 for frequency-dependent selection (or 0.4 for overdominance) would provide upper 95% confidence limits of $\Delta p_{var}$ that do not overlap the observed value of $\Delta p_{var}$ at the color locus (Table 5.3). Again, values of the selection coefficient greater than these values are no longer consistent with the observed $\Delta p_{var}$ at the color locus indicating that the observed value is not consistent with selection coefficients greater than –0.4 (or 0.4).

Figure 5.6. Graphs of $F_{ST}$ as a function of selection coefficient from simulations of (A) frequency-dependent selection and (B) overdominance. In both graphs, the arrow indicates the observed value of $F_{ST}$ from the color locus and the strength of selection which would correspond to that value of $F_{ST}$.

(A)




(B)



## DISCUSSION

The classic approach to study the evolutionary significance of a color polymorphism is to begin with the assumption that the polymorphism is selectively maintained and then to investigate the mechanisms maintaining the polymorphism. This approach has guided studies of selective mechanisms for polymorphic traits in species exhibiting color or pattern polymorphism (e.g., the pepper moth, *Biston betularia*, Kettelwell, 1961; and the land snail, *Cepaea* sp., Jones et al., 1977). However, most studies of selection on polymorphisms begin with the assumption that inter-population differences result from selection, and then study the mechanisms by which selection maintains those differences. The results clearly indicate that this approach should be revisited owing to the potential error in this assumption.

### *Evolution of the color polymorphism in R. pipiens*

In this study, I measured differences in genetic structure owing to the signature of selection at a locus controlling a green/brown dorsal color polymorphism in the northern leopard frog. Analyses comparing genetic patterns over large and small geographic ranges between neutral markers and the color locus did not significantly differ. Furthermore, temporal analysis measuring change through time did not detect differences between the genetic patterns at the color locus and at a neutral locus simulated to evolve at a rate influenced solely by genetic drift. These results indicate that genetic variation at the color locus of the

northern leopard frog does not deviate from that expected to occur owing to genetic drift. Hence, not all striking color polymorphisms may be selectively maintained. When investigating the maintenance of these polymorphisms, one should first rule out a non-selective, neutral hypothesis and thus obtain supporting evidence of selection, rather than diving straight into investigations of selective mechanisms.

The results of this study beg the question, why did two previous studies find evidence in support of a selective maintenance of the color polymorphism (see Introduction)? Both of these studies (Schueler 1979, Corn 1981) were correlational in nature, and likely reported a coincidental correlation. Moreover, the study that found variation in dates of metamorphosis among color morphs (Corn 1981) can be explained by at least one alternate hypothesis. Because Corn (1981) did not directly measure length of larval period, we do not know absolute differences between color morphs. Since females lay eggs in masses, emergence dates of siblings (of the same color) are likely to be similar. Thus, a few green females laying eggs early in the season could cause a greater percentage of early metamorphs to be green.

Do my results signify that selection is not acting on the color locus in the northern leopard frog? Not necessarily – the results only indicate that genetic variation at the color locus does not differ from expectations under the null hypothesis of genetic drift. It is possible that selection is still maintaining the color polymorphism but I did not detect it. However, if among-site stabilizing selection were maintaining the polymorphism, then I would expect genetic variation across

large distances to be larger at neutral loci than at the color locus. If each population in the study had a different equilibrium allele frequency at the color locus, we might expect increased variation among populations regardless of distance separating populations. The result of this pattern of selection would be increased genetic structure at the color locus. However, under these circumstances, one would expect to observe evidence of local adaptation by divergent allele frequencies at the color locus for populations separated by small distances.

If the green/brown color polymorphism is indeed selectively maintained, two likely scenarios could explain why I did not detect it. First, the selective mechanism maintaining the polymorphism could be something other than overdominance or frequency-dependent selection. Therefore, the simulation results, which indicated that I should detect all but very weak selection, could be based on the wrong model of selection. Under these circumstances, I cannot predict the relative strength of the selection coefficient I could detect. Additionally, it is possible that there are population differences in selection regimes through time and/or space. However, testing for such a pattern would be impossible given the wide range of expectations that could arise from such a process. Second, the selection may be so weak that although the polymorphism is actually selectively maintained, the allele frequencies at the color locus fluctuate such that allele frequency variation is indistinguishable from that caused by genetic drift. Kingsolver et al. (2001) recently reviewed the strength of selection in natural populations on quantitative traits and concluded that the median absolute value of

selection gradients for quadratic selection (i.e. disruptive or stabilizing selection) was 0.10. Additionally, Endler (1986) concluded that selection coefficients for polymorphic traits in natural populations greater than 0.1 are not rare and are probably common. However, this value may be inflated owing to an inability to account for studies not published because no evidence of selection was found (the file effect; Endler 1986).

The results from the simulations indicated the relative ability of the spatial and temporal methods to detect whether the color locus was selectively maintained, given the data. The simulations indicated that the spatial technique was much more likely to detect weak selection than was the temporal method. For my data, the temporal method requires a selection coefficient approximately 10 – 20 times greater than that for the spatial method in order for selection to be detected. This is likely because the temporal analysis only covered at most 15 generations. In order for to cause significant deviations over such a short period, selection would have to be quite strong. Over a longer number of generations, more neutral loci are likely to go to fixation while loci under balancing selection will not. Therefore, over many generations the ability to detect selection via the temporal method will be increased. In contrast to the temporal approach, the spatial approach proved able to detect relatively weak selection. Hence, the spatial approach appears to hold greater promise for future studies of selection, unless a large number of generations have elapsed between temporal samplings.

*Detecting evidence of selection on polymorphisms*

In the past, the approach to studying selection has been to begin with the assumption that inter-populational differences result from selection, and then study the mechanisms by which selection maintains those differences. Taking this approach several empirical studies have found strong evidence for balancing selection maintaining the polymorphism (e.g. Losey et al. 1997, Subramaniam and Rausher 2000, Gigord et al. 2001). Moreover, studies under artificial conditions have indicated that different morphs are likely favored under variable conditions (Borash et al. 1998, Forsman and Appelquist, 1998) and that visually oriented predators may lead to the evolution of complex polymorphic phenotypes (Bond and Kamil 1998, Bond and Kamil 2002). Although such studies have indicated how predators can influence selection on phenotype, (Endler 1986), a more systematic approach would be to first test whether one must invoke selection to explain patterns of variation in a set of populations, and *then* proceed to study the details of how selection operates.

What can I conclude about the locus comparison approach as a means to detect evidence of natural selection on single locus polymorphisms? Five studies (including this study) have attempted to measure the general signature of natural selection on a polymorphism in natural populations by comparison with putatively neutral loci among populations. Four studies analyzed selection on a color polymorphism, one on the happy-face spider (Gillespie and Oxford 1998), two on damselflies (Andres et al. 2000, Andres et al. 2002) and this study on northern

leopard frogs. The first three of these studies did indeed find evidence for selection by measuring differences in population structure at neutral molecular markers and the color locus. The fourth study investigated a single locus trait that causes a behavioral polymorphism in the fire ant, *Solenopsis invicta*. Ross et. al (1999) found that genetic structure at two protein-coding nuclear loci thought to be under selection was markedly different from the pattern of genetic structure derived from five other classes of neutral markers (allozymes, co-dominant random amplified polymorphic DNA (RAPD's), microsatellites, dominant RAPD's, and mitochondrial DNA). The take home message from this study was that genetic structure from nuclear markers, mitochondrial markers, and markers under selection all revealed patterns expected from the known features of the social and reproductive biology of *S. invicta*. These studies indicate that the locus comparison approach is applicable for a wide range of species and does not always find selective differences between morphological and molecular markers.

The locus comparison approach has two general advantages over the classic approach of measuring various mechanisms of selection as a method of identifying selective maintenance. First, the locus comparison approach is less likely to conclude that a polymorphism is selectively maintained owing to spurious correlations. Many studies in the literature report evidence of selective maintenance owing to habitat matching (e.g. Calvar and Bradley 1991, Forsman and Shine 1995), correlations with fitness traits (e.g. Shine et al. 1998), seasonal change in morph frequency (e.g. Osawa and Nishida 1992), and differential

influence of predators (e.g. Stimson and Berman 1990, Sandoval 1994). The locus comparison approach may show that in some of these species there actually is no evidence for selective maintenance (as was found for *R. pipiens*). Second, the locus comparison approach is more likely to find evidence for selection when the mechanism maintaining selection is unknown. For instance, Fincke (1994) tested various hypotheses for the selective maintenance of a color polymorphism in a damselfly and concluded that there was no evidence for the selective maintenance of the polymorphism. However, Andres et al. (2000) and Andres et al. (2002) did indeed find evidence for the selective maintenance of similar color polymorphisms in other damselflies using the locus comparison approach. Overall, the locus comparison approach provides a strong, valid method to detect even relatively weak selection in natural populations. Therefore, this method should be favored in future studies, rather than ad hoc studies of various selective mechanisms.

## CONCLUSION

Despite correlational data from two studies indicating that a striking green/brown color polymorphism in the northern leopard frog is selectively maintained, a locus comparison approach comparing genetic structure at the color locus versus genetic structure at neutral loci failed to detect evidence of weak selection. Genetic structure at the color locus, assessed both spatially and temporally, was indistinguishable from structure at neutral loci. Moreover, simulations determined that although the strength of selection would need to be

relatively strong for selection to have been detected temporally, the spatial analysis would have detected all but weak selection. This study exemplifies the importance of using the locus comparison approach to detect evidence for selective maintenance before studies attempt to measure the selective mechanisms maintaining a polymorphism.

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# CHAPTER 6: GENERAL CONCLUSIONS

The research presented in Chapters 2-5 describes the evolutionary significance of color polymorphisms and the population structure of the northern leopard frog, *Rana pipiens*, throughout the species range for both a suite of neutral molecular markers (mtDNA, microsatellites, ISSRs) and the polymorphic color locus (putatively maintained by selection). In Chapter 2 I examined the patterns found in the literature on anuran color polymorphisms. I found that current records cite at least 225 species representing 35 genera and 11 families of anurans with color or pattern polymorphisms. Of these, the mode of inheritance has been investigated in 26 species, but only conclusively demonstrated in two. Forty-six species have been described as undergoing ontogenetic change, and 32 species have been described as sexually dimorphic. The selective mechanisms maintaining these polymorphisms have only been investigated in 19 species. Finally, I conclude that the most likely mechanism maintaining these color polymorphisms is selective predation on the color/pattern itself.

In Chapter 3, I described a detailed natural history of *R. pipiens* as inferred from mtDNA inter-populational differences. My a priori expectations for patterns and processes influencing the current genetic structure of *R. pipiens* were supported by the data. However, my analyses revealed specific aspects of *R. pipiens*

evolutionary history that were unexpected. The phylogenetic analysis indicated that *R. pipiens* is split into two deeply divergent mtDNA groups, a western group and an eastern group, with the Mississippi River and Great Lakes region dividing the geographic ranges. Nested clade analysis indicated that the biological process most often invoked to explain the pattern of haplotype position is restricted gene flow with isolation by distance. Demographic analyses showed evidence of both historical bottlenecks and population expansions. Surprisingly, the genetic evidence indicated that the western region had significantly reduced levels of genetic diversity relative to the eastern region and that major range expansions occurred in both regions well before the most recent glacial retreat. This study provides a detailed history of how a widespread terrestrial vertebrate responded to episodic Pleistocene glacial events that occurred in North America. Moreover, this study illustrates how complementary methods of data analysis can be used to disentangle recent and ancient effects on the genetic structure of a species.

In Chapter 4, I described the patterns of genetic variation in Pacific Northwest populations of *R. pipiens*. I compared genetic diversity in historical and extant samples from populations in the interior and former periphery of the species' range. I found that historic peripheral populations already had reduced levels of genetic variation. Therefore, low diversity in the remnants could not be ascribed to the recent range contraction. This study shows that a common conservation strategy for rescuing genetically depauperate populations, artificial gene flow, may

be unwarranted and even detrimental to evolutionarily important peripheral populations.

In Chapter 5, I compared population genetic structure of a suite of neutral molecular markers with population structure at the color locus in *R. pipiens*. Genetic structure at the color locus, assessed both spatially and temporally, was indistinguishable from structure at neutral loci. Moreover, simulations determined that although the strength of selection would need to be relatively strong to have been detected temporally, the spatial analysis would have detected all but very weak selection. This study exemplifies the importance of using the locus comparison approach to detect evidence for selective maintenance before studies attempt to measure the selective mechanisms maintaining a polymorphism.

Several general conclusions are apparent when looking at the results of all the Chapters presented here. First, color or pattern polymorphisms remain a rich but largely unexploited system for studying the evolution of phenotypic variation in nature. Second, the results presented in this dissertation point to the need to take a combined methodological approach to understanding the complex evolutionary history of a species. This approach includes the use of multiple molecular markers, the use of multiple types of analysis, and the use of samples collected from multiple points in time. Finally, this dissertation indicated that the comparison within- and among-populations for molecular and phenotypic markers provided a powerful tool to elucidate how traits evolve.

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