Phylogeography and population genetic structure of two anuran species inhabiting the Mojave Desert

Jef Ronald Jaeger

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ABSTRACT

Phylogeography and Population Genetic Structure of Two Anuran Species Inhabiting the Mojave Desert

by

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Three studies are presented that apply the principles and methodologies of phylogeography to two anuran species (a frog and a toad) that inhabit the Mojave Desert of North America. The first study evaluates the geographic distributions of mitochondrial DNA (mtDNA) lineages (clades) within the broadly distributed toad, *Bufo punctatus*, as these relate to vicariant events hypothesized to have structured desert biotas within North America. Phylogeographic analyses support three clades with distributions corresponding to the Peninsular Desert of Baja, California, Chihuahuan Desert–Colorado Plateau (Eastern), and Mojave–Sonoran deserts (Western). Divergence levels and congruence with postulated vicariant events indicate separation of clades during the Late Neogene. Evaluation of mtDNA diversity and nested analysis reveal likely post-Pleistocene dispersal and contact across barriers separating the Eastern and Western clades. Speculation is made as to why the observed deep phylogeographic structure has not been eroded during Pleistocene interglacials.
A second study elucidates population genetic structure of *B. punctatus* among aquatic sites within mountain ranges and among mountain range groups, and relates patterns to previously determined hypotheses of population structure. Results from hierarchical analysis of molecular variance show generally high levels of genetic structure among mountain ranges, but little variation among sites within most mountain ranges. In mountain ranges demonstrating significant inter-site genetic structure, pairwise $F_{ST}$ comparisons indicate that only a few sites are responsible. A likely range expansion is inferred in two ranges. Recent convergence between two divergent lineages is indicated within one range. *Bufo punctatus* occurs primarily in patchy populations within mountain ranges, but populations appear to be isolated among ranges.

The third study focuses on the evolutionary distinctiveness of leopard frogs (*Rana* sp.) along the Colorado and Virgin River drainages within the northeastern Mojave Desert. Phylogenetic analyses of mtDNA control region sequences, restriction site variation, and congruent patterns from randomly amplified polymorphic DNA (RAPDs), indicate that these leopard frogs differ from *R. yavapaiensis*, a sister-taxon occurring in the Sonoran Desert. These two taxa show significant multivariate morphological differentiation. Leopard frogs from the Mojave Desert are phylogenetically distinct and recognizable as *R. onca*.
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CHAPTER I

INTRODUCTION

"Few patterns in ecology, evolution, conservation biology – and for that matter, most studies of biological diversity – make sense unless viewed in an explicit geographic context" (Lomolino et al. 2006).

In this dissertation, I present three studies that apply the principles and methodologies of phylogeography (Avise et al. 1987; Avise 1994, 2000) to two anuran species (a frog and a toad) within the desert environments of North America. In following the quote I use to introduce this dissertation, the topics and questions of my studies vary, but the major common themes are the geographic context in which these amphibians exist and the phylogeographic approach used to elucidate the distributions of the genealogical lineages within these amphibians. My research began in the late 1990’s during a time shortly after amphibians as a group had been recognized as in global decline (e.g., Blaustein and Wake 1990; Houllahan et al. 2000). More important to the research described herein, Avise’s (1994) book on phylogeography (Molecular Markers Natural History and Evolution) had recently been published. This was a period of expanding technological advancement and ever increasing publication rates in the field of phylogeography (Avise 2000; Riddle and Hafner 2004).
In recent years, there has been much discussion about the place, or realm, of phylogeography within the science concerned with the geographic distributions of organisms. This appears particularly the case in regards to the interface between phylogeography and historical biogeography, especially since scholars in the latter field, one with a long lineage of practitioners (Lomolino et al. 2006), appear to be attempting to redefine the scope of their science (e.g., Riddle 2005). In the following introduction, I provide a summary of the current field of phylogeography, and then place my research projects, described in the other three chapters of this dissertation, within this conceptual framework.

The Realm of Phylogeography

Phylogeography as envisioned by Avise and colleagues (Avise et al. 1987; Avise 1994) was defined as the “field of study concerned with the principles and processes governing the geographic distributions of genealogical lineages, especially those within and among closely related species” (Avise 2000, p. 3). Within the broad science of biogeography, Avise (2000) described phylogeography as a sub-discipline of biogeography, but Crisci (2001) defined phylogeography as one of nine basic approaches to the study of historical biogeography. To understand the nuances between these definitions, one must acknowledge that the field of biogeography has long been recognized as having two broad traditions (or research perspectives) – historical and ecological (Crisci et al. 2003) – predominately distinguished by different interests in the processes that govern distributional patterns of organisms and the temporal scales at which these processes function.
Phylogeography evolved as a science by integrating the concepts and principles from both macroevolution (e.g., historical biogeography, paleontology, and phylogenetics) and microevolution (e.g., demography, population genetics), and the methods of modern genomics (Avise 2000). As such, most practitioners of phylogeography make no distinction between historical and ecological biogeography. The history of a species from broad patterns to fine scale population structure is integrated through a coalescent theory perspective (Avise 2000). As often repeated, the coalescent among haplotypes or genotypes is essentially a model of lineage sorting and genetic drift run from the present backward in time to common ancestors (Harding 1996; Avise 2000). Althoff and Pellmyr (2002) explicitly summarized the sequential approach used by many phylogeographers at that time in which both traditional phylogeographic (generally tree-based analyses at a macroevolutionary scale) and population genetic approaches (often explicitly coalescent in theory) were embedded within a single study to elucidate genetic structure at various temporal and spatial scales. As stated by Riddle (2005, p. 186), “for biogeographers who have been raised on the more synthetic disciplines of macroecology and phylogeography, on the breadth and questions tractable with molecular data, and on the analytical power of phylogenetics, population genetics, and sophisticated ecological modeling, the dichotomy between ecological vs. historical biogeography simply does not track the many patterns and processes considered relevant and worthy of our attention.” In most cases, the “biogeographers” alluded to by this author would likely describe their research as “phylogeography”.

Since the early 1970s (e.g., Croizat et al. 1974), an important goal of historical biogeography has been the use of biological evidence (i.e., divergence in co-distributed
groups of species or higher taxa) to identify events in earth history, predominately
general patterns or ‘areas of endemism’ defined by vicariant events. More recent
phylogenetic biogeographers have accepted the idea that the events that assembled
regional biotas have been “so historically contingent and complex” that analysis of both
general and idiosyncratic patterns are required for explanatory reconstruction (van Veller
et al. 2003). These historical biogeographers wish to reconstruct general distribution
patterns among regional biotas and unravel the causes of idiosyncratic elements (e.g.,
dispersal) to determine reticulation between biotas (van Veller et al. 2003; Brooks 2004;
for an example see Riddle and Hafner 2004, in press). The conceptual realms of
phylogenetic biogeography and phylogeography appear to have common characteristics.
As described by Riddle and Hafner (2004, p. 95), “both approaches are concerned with
the evolutionary and geographical dynamics of speciation (or gene lineage evolution) and
biotic assembly in time and across space, and both deal analytically with problems that
arise from reticulation (e.g., between areas, biotas, or populations)”. There are several
differences between these two approaches, but what may be the main advantage of a
phylogeographic approach is the explicate addition of a temporal dimension to pattern
assessments.

Molecular clock estimates allow comparisons of the timing of divergence to the
estimated timing for the diversifying event that may have been derived from other data
(e.g., geological or paleoclimate data). The application of a molecular clock is fraught
with potential problems (e.g., Hillis et al. 1996; Edwards and Beerli 2000; Arbogast et al.
2002) caused by such factors as the differences between gene and population divergence
times, and as often practiced, estimates of the rate of molecular evolution within a taxon

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are simply applied from rates estimated in closely, or sometimes not so closely, related taxa. Ideally, molecular evolutionary rates should be calibrated using fossil dates or the timing for known geographic events that caused ancestral divergence within the taxon being evaluated (e.g., Sanderson 2002). Nevertheless, even relative evaluations of genetic divergence can be useful for testing hypotheses between deep (e.g., late Neogene) verses shallow (e.g., late Pleistocene – Holocene) diversifying events. Unfortunately, phylogeography appears to reach an empirical limit at Miocene–Pliocene timeframes because the molecular data used to estimate divergence within extant species and closely related species groups becomes increasingly saturated and less useful with time (Riddle and Hafner 2004).

Unlike historical biogeography, research in phylogeography has been overwhelmingly focused on single-taxon studies, but comparisons of genetic variation among multiple co-distributed taxa have become more common (see Riddle et al. 2000 for an early example). The general idea of ‘Comparative Phylogeography’ is that a shared biogeographic history is the most parsimonious explanation for multiple taxa exhibiting common spatial patterns in lineage subdivision (Bermingham and Moritz 1998; Arbogast and Kenagy 2001). The similarity of this idea to historical biogeography is obvious, but as practiced within a comparative phylogeographic framework, temporal congruence among patterns can be intrinsically evaluated (see review in Riddle and Hafner 2004). Separate divergence events occurring at different times or locations (e.g., repeated opening and closing of a barrier to dispersal) could produce superficially similar divergence patterns among a group of co-distributed taxa (i.e., pseudocongruence; Cunningham and Collins 1994). The elucidation of these potentially ‘contingent and
complex' histories in biotic assembly require some level of temporal assessment. The comparative phylogeographic approach also does not theoretically limit possible explanations for genealogical congruence across co-distributed taxa to historical explanations (Riddle and Hafner 2004), but appears to accept within the realm of interest other ongoing processes that may cause similar divergent patterns (e.g., broad ecological tolerance limits).

Although the comparative phylogeography approach will undoubtedly continue to grow in the future, in general practice, phylogeography has focused on studies of single species or closely related species groups, and as such the focus of this field is on understanding the origins (speciation), dispersal, and extinction of organisms. This single-species focus has been criticized by some historical biogeographers (Humphries 2000, Riddle and Hafner 2004), but the understanding of the geographic patterns intrinsic within species was a field essentially left vacant by most historical biogeographers, and the application of data from single-species studies has been responsible for breathing new life into higher-order analyses (e.g., Riddle and Hafner 2004, in press). The single-species focus and the focus of phylogeography on mtDNA (Avise 1994, 2000) has been the engine that has fostered the development and application of coalescence theory to the genetics of populations and species (see review in Avise 2000), as well as the development of sophisticated statistical approaches to coalescence based analyses of intraspecific patterns (Knowles and Maddison 2002).

One of the major impacts of phylogeography has been its application in conservation biology. As a reference to the productivity of this application, a relatively strict search of major literature (Science Citation Index) using the topic words
"phylogeography" and "conservation" revealed 74 papers published in 2004; a search abbreviating the first term to "phylogene*" reveals several multiples of this number (but the topics of some of these papers stretch the definition of phylogeography). For conservation, the predominate role of phylogeography has been to find and identify cryptic species embedded within taxonomically recognized species or species groups, and to determine important population segments for conservation (e.g., Moritz 1994a, 1994b). This usually requires identifying phylogeographic divisions within species followed by a determination of population segments with unique and independent evolutionary heritages. The interest in identifying “distinct population segments” is no doubt associated with the legal protection specifically afforded such entities, as well as “subspecies”, within the U.S. Endangered Species Act of 1973. Management also requires identification of population segments for such actions as selecting appropriate sources for translocations (Moritz 1999).

In response to the ever increasing numbers of small and fragmented natural populations, the field of conservation genetics (e.g., Smith and Wayne 1996) has expanded to address concerns that do not have a specific spatial context, such as determining the loss of genetic diversity or the effective size of a population. Most often, management concerns have been focused on microevolutionary analyses to determine the structure and dynamics of populations and to understand the conservation impacts of historical movements and degree of gene flow among populations. When these conservation studies are evaluated in a specific spatial context, they are clearly within the realm of phylogeography.
The consideration of the amount and uniqueness of genetic diversity within populations in single-species conservation planning (Moriz 1994a), or within and among co-distributed groups of species (i.e., a comparative phylogeographic approach; Moritz and Faith 1998) can be used to evaluate geographic areas so as to protect the historical patterns of independent evolution and evolutionary potential among populations within species. Along similar lines, the identification of areas that have accumulated genetic diversity are likely to be the areas that maintained populations of a species, or populations of multiple species, through periods of climatic change. Using climatic models and ecological niche modeling, one might predict which areas will maintain conditions appropriate for species of interest under future climatic predictions and overlay these patterns to identify core areas that should be protected to conserve species into the future. In a broad sense, such studies have been suggested to form a field of science described as ‘Conservation Biogeography’ (Lomolino 2004; Whittaker 2005).

Phylogeography is clearly embedded in the broad field of biogeography (Lomolino et al. 2006) but there appears be a growing sense (at least from historical biogeographers) that multiple-taxa approaches (i.e., comparative phylogeography) belong within a more inclusively defined field of historical biogeography. Nonetheless, the greatest contribution of phylogeography has been in the narrowest sense of this science, from those studies primarily focused on the spatial and temporal analysis of genealogical lineages within species and species-groups (Avise 2000). This interest in intraspecific patterns continues to be a prolific and productive area of research, particularly in the application of coalescence theory to the interface between population genetics and geography, and as an important component of conservation biology.
The Macroevolution, Microevolution, and Conservation Studies

Within this dissertation, I present three studies that span a large portion of the breadth of phylogeography covering research in both macroevolutionary and microevolutionary processes, and conservation biology. The three studies fall mostly within a more finite definition of phylogeography in that a multi-taxon approach was not specifically applied in analyses, although a preliminary data set from that presented in Chapter II was used within a publication considered an early paper in comparative phylogeography (Riddle et al. 2000). Chapters II and IV represent studies already published with several coauthors, and Chapter III represents a draft manuscript.

Although I am the lead author on these studies, I describe these studies as generally present, or expected, in the literature, thus when writing in the first person, I use the plural nominative, denoting myself and my coauthors.

Early on, I accepted an offer from my major professor, Dr. Brett Riddle, to combine a preliminary version of the phylogeographic data on *Bufo punctatus* presented in Chapter II with similar data from various co-distributed taxa derived by several colleagues in a comparative phylogeographic analysis. Our paper tested hypotheses ranging from Quaternary dispersal to late Neogene vicariance that were thought responsible for the formation of a desert biota on the Peninsula of Baja California. That paper was published in the Proceeding of the National Academy of Sciences (Riddle et al. 2000), and was considered at that time "thought-provoking" (Grismer 2000) because of its early application of a comparative phylogeographic approach. The study has led to a number of subsequent tests of our results by different research groups (e.g., Nason et al. 2002; Bernardi et al. 2003; Alvarez-Castañeda and Patton 2004). Following that
publication, I expanded the data set and analyses to broadly address the phylogeographic structure of *B. punctatus* across its distribution which is the study I present in Chapter II. I recently published this study (Jaeger et al. 2005) in the journal Molecular Ecology with Dr. Riddle and my committee member and colleague, Dr. David Bradford.

Prior to the research discussed in Chapter II, little or no recognized regional differentiation within *Bufo punctatus* had been identified across its wide distribution within the warm deserts of North America. Conceptually, this pattern suggested that *B. punctatus* was highly vagile indicating dispersal as a model to explain current distributional patterns. For example, the lack of identified regional geographic variation in populations of *B. punctatus* on the Peninsula of Baja California was used to suggest the species evolved outside of Baja California and dispersed onto the peninsula well after the formation and development of that regional desert (Grismer 1994; but see Riddle et al. 2000). As presented in Chapter II, my coauthors and I defined the geographic distributions of major mtDNA lineages embedded within this species, and evaluated these phylogeographic patterns as they relate to hypothesized Neogene vicariant events leading to the formation of desert biotas within North America (e.g., Morafka 1977; Grismer 1994; Riddle et al. 2000; Murphy and Aguirre-Léon 2002; Zink *et al.* 2000; Riddle and Hafner in press). We applied a sequential approach to the analysis (e.g., Althoff and Pellmyr 2002), and further evaluated shallow phylogeographic patterns among the major lineages by applying nested clade analysis (Templeton *et al.* 1995; Templeton 2004). From these analyses, we elucidated patterns associated with Quaternary (Pleistocene–Holocene) vicariance and dispersal within the different core desert lineages. In the
conclusion, we speculated as to why deep phylogeographic structure observed within the species has not been eroded during the multiple Pleistocene interglacials.

The research I present in Chapter III consists of a microevolutionary assessment of genetic structure in \textit{B. punctatus} from a series of mountain ranges within the northeastern Mojave Desert. This research has not yet been published, but my objective is to submit this chapter as a manuscript with little modification. My coauthors will include Dr. Riddle and Dr. Bradford, as well as Garth Spellman, a fellow graduate student and colleague who has assisted me in the analysis and interpretation of the genetic data. In the study, my coauthors and I determined the spatial scale at which genetic structure is organized and evaluated the genetic patterns to test two alternative hypotheses of population structure previously determined for this toad – nonequilibrium populations and patchy populations. These hypotheses were derived from an ecological biogeographic study (in which I participated) aimed at determining the metapopulation structure of \textit{B. punctatus} in the Mojave Desert (Bradford et al. 2003). We used the results from that study as a starting point for the research in Chapter III, namely that populations of \textit{B. punctatus} were not structured in classical metapopulations (\textit{sensu} Levins 1969) but formed either nonequilibrium or patchy populations.

The analyses presented in Chapter III relied on mtDNA control region sequence data from a large number of individuals collected from numerous sites within several mountain ranges. Assessments focused on genetic structure among ranges and among sites within ranges. To determine between the two hypothesized population models for \textit{B. punctatus} within the region, we assumed sufficient time for the genetic consequences of these population structures to become apparent (see Harrison and Hastings 1996). For
the nonequilibrium model, which implies isolated populations, we expected high measures of population divergence among areas caused by the random loss of mtDNA diversity within populations (e.g., Hartl and Clark 1997). Alternatively, for patchy populations, we expected little genetic structure among areas because the high levels of migration would limit genetic drift.

Our methods included a combination of neutrality test statistics (Tajima 1989; Fu 1997), evaluations of haplotypic diversity to determine population processes and historical patterns, and assessments of genetic structure using hierarchical analysis of molecular variance (Excoffier et al. 1992) and pairwise $F_{ST}$ calculations. Our intention was to also employ a coalescent approach, based on maximum-likelihood, to estimate migration rates among the ranges and to evaluate changes in population size through time; unfortunately, the mtDNA data failed to identify a model using two different approaches (i.e. Beerli and Felsenstein 1999; Hey and Nielsen 2004).

In the final chapter, Chapter IV, I present a conservation study that incorporates basic concepts and methods from phylogeography to identify a cryptic species and determine evolutionarily significant populations. I published this paper in the journal Copeia (Jaeger et al. 2001) with Dr. Bradford and Dr. Riddle, along with my colleague Dr. Randy Jennings who was primarily responsible for the morphological analysis presented in the study. The research focused on a rare leopard frog, *Rana onca*, which was once claimed to be extinct (Jennings 1988). Remnant populations of leopard frogs occur along the Virgin River drainage and adjacent portions of the Colorado River where these rivers enter the eastern Mojave Desert. These frogs, however, are morphologically variable, ranging from those that match the type description of *R. onca* to those that more
closely resemble *R. yavapaiensis* from areas further to the south. My coauthors and I tested two alternative hypotheses regarding the presence of these morphologically variable frogs within the region: either these frogs represent two taxa, and perhaps their hybrids; or alternatively, these frogs represent a single, morphologically variable taxon.

In this study, we used molecular and morphological evidence to evaluate these hypotheses. We based assessments on the general approach used to determine evolutionary significant units (ESU; Moritz 1994a, 1994b), and concluded with a consideration of the taxonomy of the remnant populations in light of our analyses. To evaluate mtDNA patterns, we used restriction site variation (RFLP) and control region sequences. To provide an assessment reflecting variation in the nuclear genome, we assessed randomly amplified polymorphic DNA markers (RAPD markers; Williams *et al.* 1990) and the differentiation among morphological characters. The approaches used in this study attest to the rapidly evolving methods of phylogeography, since RFLP and RAPD analyses would not be considered acceptable as major approaches for this study today, even though Chapter IV represents a paper published only 4 years ago (Jaeger *et al.* 2001). Indeed, during the time I was constructing these data sets, the university acquired automated sequencers which subsequently resulted in the mtDNA sequence data presented in this dissertation. Nevertheless, the data presented in Chapter IV adequately addressed the hypotheses of that research and the conclusions remain pertinent. This study, along with other research I participated in during my program (i.e., Bradford *et al.* 2004; Bradford *et al.* 2005), are the foundation for current efforts to develop a conservation assessment and strategy for *R. onca.*
Literature Cited


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CHAPTER II

CRYPTIC NEOGENE VICARIANCE AND QUATERNARY DISPERSAL OF THE RED-SPOTTED TOAD (*BUFO PUNCTATUS*): INSIGHTS ON THE EVOLUTION OF NORTH AMERICAN WARM DESERT BIOTAS

Abstract

We define the geographic distributions of mitochondrial DNA (mtDNA) lineages embedded within a broadly distributed, arid-dwelling toad, *Bufo punctatus*. These patterns were evaluated as they relate to hypothesized vicariant events leading to the formation of desert biotas within western North America. We assessed mtDNA sequence variation among 191 samples from 82 sites located throughout much of the species' range. Parsimony-based haplotype networks of major identified lineages were used in nested clade analysis (NCA) to further elucidate and evaluate shallow phylogeographic patterns potentially associated with Quaternary (Pleistocene–Holocene) vicariance and dispersal. Phylogenetic analyses provided strong support for three monophyletic lineages (clades) within *B. punctatus*. The geographic distributions of the clades showed little overlap and corresponded to the general boundaries of the Peninsular Desert, and two continental desert regions, Eastern (Chihuahuan Desert–Colorado Plateau) and Western
(Mojave–Sonoran deserts), geographically separated along the Rocky Mountains and Sierra Madre Occidental. The observed divergence levels and congruence with postulated events in earth history implicate a Late Neogene (latest Miocene–Early Pliocene) time-frame for separation of the major mtDNA lineages. Evaluation of nucleotide and haplotype diversity and interpretations from NCA reveal that populations on the Colorado Plateau resulted from a recent, likely post-Pleistocene, range expansion from the Chihuahuan Desert. Dispersal across historical barriers separating major continental clades appear to be recent, resulting in secondary contacts in at least two areas. Given the observed contact between major clades, we speculated as to why the observed deep phylogeographic structure has not been eroded during the multiple previous interglacials of the Pleistocene.

Introduction

In his biogeographical analysis of the herpetofauna of the Chihuahuan Desert, Morafka (1977) presented a general model for the evolution of North American desert biotas that promoted the importance of two major pre-Pleistocene vicariant events in the diversification of a widely distributed, ancestral desert biota. These vicariance events were the formation and uplift of the Colorado Plateau–Sierra Madre Occidental (roughly the Continental Divide) separating the Chihuahuan Desert from the Sonoran and Mojave deserts, and the expansion of the Sea of Cortés, separating the peninsula of Baja California from the continental mainland. Morafka (1977) hypothesized that the closure of a last portal of desert habitat between the Chihuahuan and Sonoran deserts was largely caused by climatic cooling starting approximately 3 million years ago (Ma).
Concurrently, landscape and climatic changes in mainland desert regions near the head of the Sea of Cortés resulted in the development of the modern biotic patterns in that region. While the details of his models have been modified or refined, particularly the model associated with development of the Peninsular Desert biota (e.g., Grismer 1994; Riddle et al. 2000a; Murphy & Aguirre-Léon 2002), Morafka (1977) articulated a new paradigm. This perspective countered previous scenarios that emphasized Pleistocene glacial-interglacial cycles as the dominant force in the evolution of North American desert biotas (e.g., Savage 1960; Findley 1969; Hubbard 1973).

Support for the hypothesis of pre-Pleistocene vicariance in the structuring of North American arid-adapted biotas has come from a greater understanding of the geological and climatic processes that formed the deserts and empirical observations of phylogeographic patterns (Hafner & Riddle in press; Riddle & Hafner in press). Numerous widespread species and species-groups consist of two or more genealogical lineages that are sufficiently divergent to be consistent with postulated pre-Pleistocene vicariance. Many of these taxa have congruent phylogeographic patterns that are generally coincident with the boundaries of the Chihuahuan, Sonoran-Mojave, and/or Peninsular deserts (Table 2.1).

Morphologically defined species with distributions spanning two or more of these desert regions have often been explained by, and taken as evidence for, Late Pleistocene dispersal (Riddle & Hafner 1999). For example, Grismer (1994) stated that there is a lack of strong morphological difference between populations of the red-spotted toad, *Bufo punctatus* Baird & Girard 1852, on the peninsula of Baja California and on the continental mainland. He saw this pattern as evidence that this widespread, arid-dwelling
toad evolved on the mainland and dispersed onto the peninsula relatively recently (Pleistocene–Holocene), well after Pliocene vicariance had led to the evolution of other desert taxa unique to the peninsula. Under this interpretation, *B. punctatus* would provide evidence of biotic reticulation between the Peninsular and Sonoran-Mojave deserts, obscuring the distinction between the biotas of these regions. This perspective was later rejected by Riddle et al. (2000a) when peninsular populations of *B. punctatus* were found to comprise a single monophyletic mtDNA lineage divergent from lineages on the continental mainland. The observed phylogeographic distribution and deep level of sequence divergence were congruent with patterns evident within several other taxa, and were determined to be more consistent with postulated Pliocene vicariance than with more recent dispersal scenarios.

As with the development of the Peninsular Desert biota, the separation of Chihuahuan and Sonoran Desert biotas appears to be the result of multiple episodes of isolation and divergence throughout the Late Neogene (e.g., Zink et al. 2000; Riddle et al. 2000c). The uplift of the Sierra Madre Occidental, Mexican Plateau, and Colorado Plateau (Hafner & Riddle in press) during the Late Miocene–Early Pliocene, potentially in association with early shifts towards colder climates (Morafka 1977), appears to have been a fundamental vicariant event leading to early biotic diversification within the continental deserts.

*Bufo punctatus* is broadly distributed across warm arid and arid-subtropical regions of southwestern North America (Fig. 2.1). It occurs throughout the Deming Plains (Cochise filter barrier) between the Sonoran and Chihuahuan deserts (Morafka 1977). This narrow desert corridor between the Sierra Madre Occidental and Colorado
Plateau may have been ephemeral during the Pleistocene (Morafka 1977). During the last glacial maximum (Wisconsin glacial, 18,000 years ago; Ka), this region of the Chihuahuan Desert was a pinyon-juniper-oak (\textit{Pinus}, \textit{Juniperus}, and \textit{Quercus}) woodland and apparently only transformed to arid-grassland and desertscrub vegetation in the middle Holocene, as recently as 4 Ka (Van Devender et al. 1984; Van Devender 1990). The lack of readily recognized morphological differentiation in populations of \textit{B. punctatus} on either side of the Deming Plains suggests the possibility of Late Quaternary dispersal between the Sonoran and Chihuahuan deserts (i.e., range expansion from one desert region to the other) or repeated contact between regional populations (i.e. migration). Either process should have left a signature of reduced genetic divergence among the regional desert populations. Riddle et al. (2000a), however, revealed a deep east-west mtDNA divergence between continental populations of \textit{B. punctatus}. Although this pattern implicated pre-Pleistocene divergence, samples were insufficient for further assessment.

Evidence from pollen records and packrat middens has documented substantial changes in the distributions of desert plant assemblages within North America during the latest glacial-interglacial cycle (Bentancourt et al. 1990; Thompson & Anderson 2000). Presumably, these biotic responses to climatic change reflected similar distributional responses during the multiple climatic oscillations of the Late Quaternary (about the last 700 ka; Webb & Bartlein 1992). The regional deserts that existed during interglacials, and may have reached their maximum extent during the Holocene, were more limited in distribution during glacial periods. To the degree that niche requirements of \textit{B. punctatus} have remained stable over time (e.g., niche conservatism; Wiens 2004), we predict that
the distribution of this species would have tracked shifts in its habitat distribution through glacial cycles. Such distributional shifts need not erode phylogeographic structure produced by earlier episodes of isolation and divergence, if range shifts are constrained in spatial extent by abiotic or biotic factors (Riddle 1998). Indeed, elucidation of population responses to the most recent episode of climatically induced habitat changes might provide insight into the mechanisms that have maintained deeper-scale phylogeographic pattern in the face of multiple glacial-interglacial cycles.

Herein, we expand on the earlier phylogeographic analysis of *B. punctatus* and evaluate mitochondrial DNA (mtDNA) sequence data to further define the geographic distributions of embedded maternal lineages. We evaluate these patterns as they relate to hypothesized vicariant events associated with the early formation of North American deserts. Phylogeographic structure associated with very recent events may be more readily interpreted with approaches that do not assume *a priori* that genetic architecture is captured within bifurcating phylogenetic trees (Althoff & Pellmyr 2002). We use nested clade analysis (NCA; Templeton et al. 1995; Templeton 2004) and a neutrality test statistic to further evaluate patterns within major identified clades to infer geographic population structure and to identify potential responses (e.g., range expansion, fragmentation) to Late Quaternary habitat changes.

Materials and Methods

Samples

Samples of *B. punctatus* were field collected or acquired from university collections or other researchers (see Appendix). A total of 192 *B. punctatus* were
sampled from 82 locations representing the majority of the species distribution (Fig. 2.1),
except for portions of the range in the lower Sonoran and Chihuahuan deserts in Mexico.
*Bufo debilis* and *B. retiformis* were selected as outgroups for phylogenetic analyses based
on a hypothesis of phylogenetic relationships (Ferguson & Lowe 1969) and support from
interspecific mtDNA analyses that consistently demonstrated some alliance between *B.
punctatus* and a clade containing *B. debilis* and *B. retiformis* (Graybeal 1997).

**Laboratory Methods**

Total genomic DNA was extracted from heart, muscle, liver, toe tissue, and in a
few cases frozen hemolyzate using standard phenol-chloroform extraction. The
mitochondrial gene cytochrome b (cyt b) was selected for genetic analysis. This gene has
an evolutionary rate within bufonids that is useful for analyses within Late Cenozoic
time-frames (Graybeal 1993, 1997). A portion of the cyt b gene was amplified by
polymerase chain reaction (PCR) method using primers MVZ43 (Graybeal 1993) and a
slightly modified version of MVZ16 (Moritz et al. 1992). Generally, PCR was performed
in 50 μL reactions using approximately 30–200 ng of total genomic DNA, 1.2 U
Amplitaq polymerase (PE Applied Biosystems, Inc.), and a final concentration of 3 mM
of MgCl₂ in a standard reaction mix (50 mM KCl, 10 mM Tris-HCl, 0.001% gelatin, 0.5
μM each primer, 0.2 mM each dNTP). Thermal cycling was accomplished using a 55 °C
annealing temperature. PCR fragments were purified using GeneClean (II Kit, BIO 101,
Inc.).

Fluorescence-based cycle sequencing was conducted on the purified PCR
templates using ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit
chemistry (PE Applied Biosystems, Inc.) with unincorporated dye labels removed by

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ethanol precipitation (PE Applied Biosystems, Inc. manufacturer protocol) or by Sephadex gel separation (e.g., Sambrook et al. 1989). Sequencing primers consisted of those used in PCR and two internal primers designed specifically for sequencing B. punctatus cyt b (Riddle et al. 2000a). Electrophoresis and visualization of sequences were completed on an ABI Prism 310 automated sequencer (PE Applied Biosystems, Inc.).

Phylogenetic Analyses

Sequence alignments were made by eye and sequences checked for nucleotide and reading frame accuracy using BioEdit (version 5.0.9; Hall 1999). Haplotypes were identified by calculating the number of nucleotide differences among sequences assuming pairwise deletion of ambiguous sites (< 0.017 of any sequence was ambiguous). Unique haplotypes were sequenced a second time for the complement to confirm observed patterns.

Tree topologies were generated using maximum likelihood (ML), Bayesian inference (BI), and maximum parsimony (MP) on a subset of the data in which identical haplotypes were merged. Modeltest (version 3.06; Posada & Crandall 1998) was employed to assist in selection of an appropriate model of sequence evolution for ML and BI analyses (56 nucleotide substitution models tested, default option neighbor-joining starting tree under a Jukes-Cantor substitution model). The HKY model (Hasegawa et al. 1985) with among-site rate variation approximated by gamma distribution was determined by hierarchical likelihood rate test to be the best-fit model for the data.

For ML analysis, the associated model parameters from Modeltest were subsequently used in a heuristic search (random stepwise addition, 10 replications, 1 tree
held at each step, tree-bisection-reconnection branch-swapping, and collapsing zero
length branches) as implemented in PAUP* (version 4.0b10; Swofford 2000). Model
parameters were then re-estimated from the resulting trees and applied in a subsequent
heuristic search using the original ML trees as starting trees.

Bayesian inference of phylogeny was implemented using MrBayes (version 3.0b4;
Huelsenbeck & Ronquist 2001). Three runs of $1.05 \times 10^6$ generations were conducted
(sampling every 100 generations, 50,000 generations discarded as burn-in). Model
parameters were estimated as part of the analyses assuming flat Dirichlet priors for
substitution rates and nucleotide frequencies, a wide uniform distribution for the gamma
shape parameter, and a flat Beta for the transition/transversion ratio (default settings). To
improve the rates of chain swaps during analyses, the temperature difference between
chains was lowered to 0.05, although this made little difference in final results. Multiple
runs, each starting with a random tree, were used to detect any substantial differences in
analyses that could indicate instability. Posterior probabilities, presented as support for
phylogenetic patterns, were derived as the average of all sampled BI trees (30,000
sampled trees).

Unweighted MP analysis was implemented in PAUP* by employing the heuristic
search algorithm under the same search parameters as in the ML analysis. The robustness
of resulting MP topologies was assessed by nonparametric bootstrap (1000 replicates)
under the same heuristic search criteria.

Estimates of net sequence divergence among the lineages of *B. punctatus*,
corrected for variation among haplotypes within each of the lineages (e.g., Edwards
1997), were calculated as p-distances (pairwise deletion) in MEGA2 (Kumar et al. 2001).
Haplotype and nucleotide diversity (Nei 1987) were estimated using ARLEQUIN (vers. 2.000; Schneider et al. 2000). Rate constancy (molecular clock) among all haplotype sequences was evaluated using a likelihood ratio test in which a rate constancy constraint was enforced on the ML trees (implemented in PAUP*) and the resulting likelihood score compared against the value derived from the original ML trees (Felsenstein 1981, 1988).

Nested Clade Analyses

We conducted NCA (Templeton et al. 1995; Templeton 1998, 2004) for each of the major clades. Haplotype networks (Smouse 1998; Posada & Crandall 2001) used a parsimony algorithm (Templeton et al. 1992) and 95% probability criterion for connections as implemented in the program TCS (version 1.6, Clement et al. 2000). Resulting networks were converted into a hierarchical nested design following the rules of Templeton et al. (1987) and Crandall (1996). Haplotype hierarchical position and geographical locations (latitude and longitude) were then incorporated into NCA using the program GeoDis (Posada et al. 2000). Null distributions for permutational contingency table tests comparisons were generated from 10,000 random permutations and considered significant at $\alpha = 0.05$. NCA statistics (i.e., $D_c$, $D_n$, I-T) were then jointly evaluated for their fit to expectations from various models of population structure and historical events (Templeton et al. 1995; Templeton 1998) using a reference key (Templeton 2004). Inferences were considered significant for only those clades in which the null distribution (i.e., a random association between haplotypes and geography) were rejected in the contingency table tests. Inferences of population expansion from NCA were corroborated by Fu’s $F_s$ test as implemented in ARLEQUIN. Fu (1997) has shown that $F_s$ is a powerful test for detecting population growth, and in the absence of selection,
strongly negative values are expected from population expansion. Critical values for the tests were considered significance at $P \leq 0.02$ (Schneider et al. 2000).

Results

Phylogenetic Analyses

A total of 49 haplotypes of *B. punctatus* were identified from 666 basepairs of sequence data examined for all samples. In total, 171 sites varied, of which 132 were parsimony informative. The variable sites comprised 7% (15) of first codon positions, 2% (4) of second codon positions, and 68% (152) of third codon positions. Among the haplotypes of *B. punctatus*, 108 sites varied, 94 of which were in third codon positions. No premature stop codons were observed. Sequences demonstrated a strong light strand bias against guanine which is characteristic of bufonid mtDNA (Graybeal 1993, 1997; Macy et al. 1998; Mulcahy & Mendelson 2000). The pairwise number of nucleotide differences among haplotypes of *B. punctatus* ranged from 1 to 56 (p-distances $\leq 0.0841$). Between the haplotypes of *B. punctatus* and the two outgroup taxa, the number of nucleotide differences ranged from 100 to 108, and from 103 to 116, respectively (p-distances 0.1502 to 0.1742).

Under the estimated model parameters from Modeltest, four equally likely ML trees were derived and the following model parameters were estimated from those trees (transition/transversion ratio = 13.4471; base frequencies, $A = 0.27610$, $C = 0.30567$, $G = 0.13774$, $T = 0.28049$; $\alpha$ shape parameter = 0.13208). The second iteration analysis (rearrangements evaluated = 214,816) found four equally likely ML trees ($-\ln L = 2189.31148$). All four ML trees shared the same major topology in depicting three distinct clades within *B. punctatus*. The most conservative tree depicted the association...
between the major clades as a polytomy (Fig. 2.1). The other trees differed predominately in the order of branching among the major clades (with each of the major lineages being shown as branching earlier than the others in one of the three trees). Branch lengths associated with the patterns among major clades were diminutive.

Tree reconstructions under BI converged on similar average likelihood values in all runs (-\(\ln L\) = 2255.31, 2257.45, 2259.40, respectively), with consensus trees (50\% majority rule) revealing the same three major clades depicted in the ML analysis. Posterior probabilities (PP) revealed strong support for the major clades (PP \(\geq 0.99\) per clade). As in the ML analysis, the BI consensus tree indicated a polytomy between the major clades with no support for any particular branching order (Fig. 2.2).

Unweighted MP resulted in 190 trees (score = 261), with the strict consensus depicting the same three major clades as in the other analyses (Fig. 2.2). Bootstrap support values (BS) for these clades under the MP criteria were high (BS = 100 per clade). The MP analysis indicated a particular branching order among the clades (Fig. 2.2); however, there was very little support (BS = 52) for the indicated topology.

Evolutionary rate constancy was marginally rejected in likelihood ratio tests of the ML trees with and without a molecular clock constraint (approximate \(\chi^2 = 69.28, df = 49, P = 0.03\)). A further likelihood ratio test on a ML tree containing only haplotypes of \textit{B. punctatus} did not reject the hypothesis of rate constancy (approximate \(\chi^2 = 62.09, df = 47, P = 0.07\)). These borderline results imply a lack of evidence for substantial rate heterogeneity among the sequences.

The geographic distributions of haplotypes within the three major clades exhibited very little overlap. Two continental clades, generally eastern and western in distribution,
and a clade concordant with the boundaries of the Peninsular Desert were identified. Henceforth, these three major clades are referred to as the Eastern, Western, and Peninsula, respectively (Fig. 2.2). Sequence divergences among these clades (adjusted for within clade variation) were all very similar (p-distances: Western–Eastern = 0.0669; Western–Peninsular = 0.0683; Eastern–Peninsular = 0.0670). This level of divergence was lower than that observed between the recognized sister-taxa B. debilis and B. retiformis included in this study (= 0.0796). Sequence divergences within clades were about an order of magnitude lower (Western = 0.0084; Eastern = 0.0070; Peninsular = 0.0039).

Geographic Distribution of Major Clades

The continental clades were geographically separated predominately along the Rocky Mountains and Sierra Madre Occidental (roughly the Continental Divide). Haplotypes in the Eastern clade occurred throughout the Colorado Plateau and northern regions of the Chihuahuan Desert, and extended into the grassland regions of eastern New Mexico and western Texas. Haplotypes of the Western clade occurred throughout the Mojave and Sonoran Deserts. Within the eastern Mojave Desert near the transition to the Colorado Plateau, Eastern and Western haplotypes were found in sympatry at sites near the confluence of the Colorado and Virgin rivers (now the Overton Arm of Lake Mead; Fig. 2.1, locations 4 and 5). Within the region of contact between Sonoran and Chihuahuan deserts, Eastern and Western haplotypes occurred in sympatry within the Peloncillo Mountains (Fig. 2.1, location 42), a long recognized point of contact between these desert biotas (e.g., Dessauer et al. 1962; Findley 1969; Morafka 1977).
No sites of sympatry were located among haplotypes from Peninsular and Western clades, but our sampling within the border region of the Peninsular and Sonoran deserts was insufficient to localize an area of contact, if one exists. Several Peninsular Desert taxa reach their northern limits in the Peninsular Mountain Ranges of southern California (Murphy 1983; Grismer 1994). The distribution of the Peninsular clade of *B. punctatus* does not appear to extend northward into this region where we found only haplotypes from the Western clade (Fig. 2.1, locations 25–27).

Nested Clade Analyses

Nested clade analysis of the Eastern clade was based on 19 haplotypes identified within 79 samples collected from 33 locations and the haplotype network and nesting design presented in Fig. 2.3A. Contingency table tests determined significant nonrandom association between sampling locations and Eastern clades 2-1, 2-2, and for the total Eastern cladogram (all $P$ values $\leq 0.05$). Interpretations of distance measures and relative haplotype/clade ages (i.e., interior verses tips) using the inference key revealed a significant signature of contiguous range expansion within the Chihuahuan Desert–Colorado Plateau region (Fig. 2.4A). The significantly negative Fu’s $F_s$ statistic value for the Eastern Clade (Table 2.2) supported the inference that this population has undergone substantial growth. This range expansion was particularly conspicuous within Eastern clade 2-1 with haplotypes from Eastern clade 1-1 found throughout most of the region but comprising all samples from the Colorado Plateau west of the Rio Grande (Fig. 2.1, locations 4, 5,63–72; Fig. 2.3B). In this region of the Colorado Plateau, haplotypes were mostly identical (i.e., E01) with a few derived haplotypes that differed from the common haplotype by a single basepair (Fig. 2.3A). A significantly negative $F_s$ statistic value,
consistent with population expansion (Table 2.2), was derived from the samples in this region, whereas the samples from the Chihuahuan Desert (Fig. 2.1, locations 42–62) showed a non-significant $F$, statistic value, indicating that the major population growth within the Eastern clade occurred on the Colorado Plateau. Allopatric fragmentation was implicated for clades within Eastern clade 2-2, but this signal should be interpreted with much caution since it is predominately derived by the significant geographical restriction of Eastern clade 1-4 which is represented by only three samples from a single location.

The haplotype network and nesting design of the Western clade NCA (Fig. 2.5A) were derived from 16 haplotypes identified within 88 samples from 42 locations. We excluded the 3 samples from the southern Sonoran Desert because of the large geographic distance separating them from the more densely sampled populations in the northern Sonoran and Mojave deserts. Contingency table tests determined significant nonrandom association between sampling locations and Western clades 2-2, 2-3, and the total Western cladogram (all $P$ values $\leq 0.001$). The only conclusive inference from this NCA, however, was a signal of contiguous range expansion at the total clade level (Fig. 2.4B). The interior status of Western clade 2-2, necessary for inference at the total clade level, can be weakly inferred from the phylogenetic analyses. All haplotypes within this clade appear on trees to have diverged earlier than haplotypes within the other Western clades (Fig. 2.2), thus indicating that clade 2-2 may well contain the oldest haplotypes. Although there is a tendency for haplotypes of high frequency to be near the root of a genealogy (Castelloe & Templeton 1994), the most common haplotype in this analysis (W05), as well as the most common haplotype in the Eastern clade NCA (E01), both appear to have
resulted from participation in range expansions. The high frequencies of these haplotypes in the data sets may be artifacts of the expansion process and our sampling.

An inference of population expansion for the Western clade was not supported by Fu’s $F_s$ test, although the test statistic was strongly negative (Table 2.2). Population growth within the northeastern Sonoran Desert, south of the Gila River (Fig. 2.1, locations 36–42), was supported by a significantly negative $F_s$ statistic value (Table 2.2). The lack of diversity in this region appears to have been a major factor in the inference of contiguous range expansion inferred from the NCA at the total Western clade level.

Within Western clade 2-1, a signature of past fragmentation was implicated in the geographic separation between Western clade 1-1 (haplotype W08), found in populations within the eastern Mojave Desert of California and Nevada, and Western clades 1-2 and 1-3 (haplotypes W01 and W11, respectively) in populations within the Peninsular Ranges of southern California (Fig. 2.5B). Contingency table analysis, however, failed to reject the null hypothesis of random association between geographic location and haplotypes within these clades.

The NCA of the Peninsular clade was based on 11 haplotypes identified within 22 samples from 8 locations (Fig. 2.6A, B). No patterns were supported in the contingency table tests of these data (all $P$ values > 0.05; nested-clade statistics not shown), but a strongly negative $F_s$ statistic value (Table 2.2) indicated the possibility of population growth for the Peninsular clade. There also appeared to be a north-south trend in haplotype structure (Fig. 2.6B). Further sampling, however, is needed to assess these patterns.
Discussion

Pre-Pleistocene Vicariance

The patterns of genealogical relationships suggest a near simultaneous divergence among the major clades of *B. punctatus*. Sequence divergences among the three major clades were almost identical, and phylogenetic analyses revealed no significant support for any particular branching order among these clades (Fig. 2.2). Haplotypes from these clades showed little overlap in their geographic distributions (Fig. 2.1). We propose that this phylogeographic pattern was likely initiated by two Late Neogene vicariant events— the early stages in the development of the peninsula of Baja California, and the uplifting of the Colorado Plateau, Mexican Plateau, and the Sierra Madre Occidental.

The rate of molecular evolution for cyt b appears to be slower in bufonids than in other vertebrates (Graybeal 1997). Bufonid mtDNA evolution has been estimated (Macy et al. 1998) at about 1.38% change between lineages per million years for a region consisting predominately of the ND1 gene (subunit of the NADH dehydrogenase). This rate was based on divergence levels between Asian and European bufonids and the estimated time for the aridization of Central Asia caused by the uplifting of the Transhimalaya and Tibetan Plateau (Macy et al. 1998). There is evidence, within the taxa used to derive this estimate, that the rate of evolution for cyt b is roughly similar to that for ND1. Estimated pairwise divergence between *B. gargarizans* (from the eastern Tibetan Plateau) and *B. viridis* (a European species closely related to the *B. bufo* species group) averaged 17.3% for 519 basepairs of cyt b (Liu et al. 2000) while the ND1 region differed by 16.2% (Macey et al. 1998). Although the evolution of cyt b may occur at a slightly faster pace, the rates between these two genes are roughly comparable. While a
molecular clock should be used with caution to estimate times of lineage divergence (Hillis et al. 1996; Edwards & Beerli 2000; Arbogast et al. 2002), the application of the rate indicated above to the observed divergence levels within \textit{B. punctatus} implicate a latest Miocene to Early Pliocene time-frame for the separation of the major mtDNA lineages.

Riddle et al. (2000a) provisionally attributed the divergence between populations of \textit{B. punctatus} on the peninsula of Baja California and those on the continental mainland to a marine barrier that isolated the peninsula during the Late Pliocene (the northern gulf vicariance, about 3 Ma). To be consistent with this hypothesized timing, the evolutionary rate for cyt b in \textit{B. punctatus} would need to be near, or in excess of, 2% change between lineages per million years, assuming the time of gene lineage coalescence is not appreciably different from time of lineage divergence. Perhaps, the northern gulf vicariance is older than generally referenced (see Murphy & Aguirre-Léon 2002) allowing for a time-frame more consistent with the cited rate of bufonid mtDNA evolution. Alternatively, the Peninsular clade could have split from continental clades during the latest Miocene to Early Pliocene (> 4 Ma) development of the Sea of Cortés (southern gulf vicariance; Grismer 1994; Riddle et al. 2000a; Murphy & Aguirre-Léon 2002). A number of primarily warm desert taxa, including \textit{B. punctatus}, have distributions that extend southward from the Sonoran Desert into the seasonally deciduous forests of Sonora and Sinaloa (Hafner & Riddle in press). The southern gulf vicariance has been hypothesized to include members of a subtropical thornscrub biota (Grismer 1994). If these thornscrub forests represent an ancestral habitat for \textit{B. punctatus}, then this toad could have been included within a southern gulf vicariant event – a hypothesis that is
plausible given the estimated ages of divergence between the mainland and peninsular clades.

The deep level of sequence divergence between Eastern and Western continental clades of *B. punctatus* and the geographic separation of these clades along the axis of the Rocky Mountains and Sierra Madre Occidental is consistent with vicariance of Sonoran and Chihuahuan desert biotas initiated by the secondary uplift of the Sierra Madre Occidental and Mexican Plateau (see Riddle 1995). The hypothesized Late Pliocene timing for an effective closure of arid habitats caused by the onset of climatic cooling (Morafka 1977) may be too recent to explain the observed level of divergence between lineages of *B. punctatus*. Earlier vicariance (Late Miocene–Early Pliocene) between Sonoran and Chihuahuan desert populations directly associated with orogenesis of the Sierra Madre Occidental and uplift of the Mexican Plateau presents a plausible alternative.

**Pleistocene – Holocene Patterns**

Secondary contact of continental clades within the eastern Mojave Desert is likely a recent occurrence (Late Pleistocene–Holocene). Very low haplotype and nucleotide diversity throughout the Colorado Plateau west of the Rio Grande (Fig. 2.3; Western Colorado Plateau in Table 2.2) is consistent with an interpretation that *B. punctatus* colonized this area recently and rapidly. The NCA inference of contiguous range expansion for haplotypes within this region supports this interpretation, as does the signal of population growth derived from Fu’s $F_s$ test. Reconstructions of Pleistocene macrohabitats within the Southwest (Lomolino et al. 1989; Bentancourt 1990; Thompson et al. 1993; Thompson & Anderson 2000) indicate that arid vegetation assemblages,
currently inhabited by *B. punctatus*, did not persist on most of the Colorado Plateau during the latest glacial period (Wisconsin). The Grand Canyon of the Colorado River forms a low-elevation corridor extending from the west into the higher-elevation Colorado Plateau. Even lower elevations within the canyon were mostly occupied at that time by a pluvial desertscrub comprised principally of species from the Great Basin, which typically dominate in areas of greater seasonality (Cole 1990).

The current distribution of *B. punctatus* within the Mojave Desert provides evidence of climatic limits for this toad that reinforce interpretations that the Colorado Plateau was unoccupied during the Wisconsin glacial maximum. *Bufo punctatus* currently occurs within lower bounds of pinyon-juniper woodlands on mountain slopes at elevations where desert plants common to the Great Basin also occur. However, its northern limit is reached well below latitudes in which Great Basin vegetation becomes dominant. This climatic limit was clearly evident in a study of habitat patch occupancy by *B. punctatus* within the eastern Mojave Desert (Bradford et al. 2003). In that study, strong negative relationships were documented between patch occupancy and increases in both elevation and latitude. Given the current climatic limits of *B. punctatus* and the latitudinal and elevational shifts in climatic conditions during the Wisconsin, it seems unlikely that any area of the Colorado Plateau was continuously occupied by this species.

In contrast, the Mojave Desert contains low elevation areas that appear to have mitigated colder climatic conditions during recent glacial periods. Death Valley and the Amargosa River drainage in the northern Mojave Desert both appear to have retained desertscrub vegetation during the Wisconsin, but this assemblage contained numerous species common to colder regions (Spaulding 1990). The area of the lower Colorado
River, extending northward into southern Nevada, appears to have maintained more desert-like conditions (Betancourt et al. 1990; Thompson & Anderson 2000). Haplotype and nucleotide diversity for *B. punctatus* are much higher in the Mojave Desert (Fig. 2.1, locations 1–21, 28) than on the western Colorado Plateau (Table 2.2) likely reflecting Pleistocene persistence of populations in the Mojave Desert.

Bradford et al. (2003) suggested that the Amargosa River and the lower Colorado River were the sources for post-Pleistocene expansions of *B. punctatus* into the surrounding areas. There is some support for this perspective in the form of east-west structuring of haplotypes between populations on the lower Colorado River in Nevada (clade 2-2; Fig. 2.5) and populations in mountains further to the west (clade 1-1; Fig. 2.5). There is also evidence of past fragmentation between clades centered in the Mojave Desert (clade 1-1; Fig. 2.5) and those in the extreme northwestern edge of the Sonoran Desert (clades 1-2, 1-3; Fig. 2.5), which could be attributed to Pleistocene refugia patterns. None of these patterns, however, have statistical support from initial permutational contingency table tests in the NCA.

We interpret the pattern of low haplotype and nucleotide diversity on the Colorado Plateau as provisional support for rapid post-Pleistocene range expansion from populations in the northern Chihuahuan Desert. The most likely scenario is that as the climate warmed, toads within the Rio Grande rift valley, or expanding northward up the valley, jumped the continental divide into the San Juan or Little Colorado River systems. Toads then quickly expanded throughout the Colorado Plateau along the major river corridors. This range expansion is consistent with a leading edge, or pioneer model (Hewitt 1993) of long distance dispersants establishing rapidly expanding populations of

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low genetic diversity within newly colonized areas (reviewed in Hewitt 1996, 1999). The spread of Eastern haplotypes towards the Mojave Desert may have moved quickly along the southwestward flowing Colorado River, facilitated by passive dispersal of adults and larvae. Expansion of Eastern haplotypes into the Mojave Desert may now be limited as immigrants are entering regions occupied by established local populations (Hewitt 1993). Eastward dispersal of Western haplotypes up-river into the Grand Canyon was likely hindered by river flow and topography.

Maintaining Vicariant Structure in the Face of Pleistocene Climate Oscillation

The current contact between major continental clades of *B. punctatus* in at least two localities begs the question: Why has deep phylogeographic structure not been eroded during the multiple climatic oscillations during the Pleistocene period of the last two million years, or more specifically, during the more extreme glacial-interglacial climatic cycles (Webb & Bartlein 1992) of the last 700,000 years? Seemingly, if current climatic conditions exemplify previous interglacial climates, contact between clades of *B. punctatus* would have occurred multiple times through the past million years. We could account for the lack of empirical evidence for regional introgression of haplotypes between clades by two scenarios.

Perhaps arid habitats were less extensive during previous interglacials. If true, then the clades of *B. punctatus* may not have actually come into contact during the Pleistocene and the current contact may be unique. The expansion of Eastern clade haplotypes through the Colorado Plateau and into the eastern Mojave Desert appears to be a Holocene event. The apparent recent expansion of the Western clade into the northeastern Sonoran Desert and northwestern fringe of the Chihuahuan Desert may
potentially be explained by recent development of favorable desert conditions within this region. Evidence from packrat midden data (Van Devender et al. 1984) indicates that the development of arid conditions in the northern Chihuahuan Desert following the Wisconsin glacial did not occur until the mid to late Holocene (~ 4 Ka). Unless climatic conditions were similarly developed during previous interglacials, aridization of areas peripheral to the core deserts may not have occurred. Under conditions less climatically or ecologically favorable, *B. punctatus* may not have been able to overcome barriers between desert regions during previous interglacials. A detailed evaluation of the distribution of arid habitats in these regions across multiple interglacial cycles would provide insight into this possibility, but we know of no paleoecological records currently available of sufficient resolution.

Alternatively, the mixing of maternal lineages among regional desert populations of *B. punctatus* may have occurred during past interglacials, but if dispersal of clades did not expand beyond climatically-fringe desert regions, a return of colder climates could have simply eliminated the intermixed populations (Hewitt 1996). Extirpation of introgressed populations would have maintained the integrity of the major lineages within each of the core desert regions. The dispersal of clades among the deserts may have been geographically limited by numerous factors (reviewed in Hewitt 1996, 1999), including intraspecific competition between expanding populations dispersing from each of the core desert regions. A similar scenario was presented to explain a purported lack of phylogenetic structure within Pleistocene large mammalian populations in Europe when current populations of many of these species show strong phylogeographic signals associated with recent expansion from glacial refugia (Hofreiter et al. 2004). The loss of
contact populations may not have been necessarily limited to temporal scales associated with glacial-interglacial oscillations. Fluctuations in climatic conditions on a temporal scale of centuries to millennia (Roy et al. 1996) could have effectively retarded expanding desert populations in marginal habitats if warm interglacial climates are interrupted by short-term cold conditions (Hewitt 1996).

_Bufo punctatus_ as a Member of the Warm Deserts Biota

A profound change in our understanding of the biogeography of North American warm deserts began with the notion that widespread taxa might have been isolated by tectonically-driven landscape transformations (e.g., Morafka 1977; Murphy 1983). These alternatives to scenarios dominated by Pleistocene climatic responses were not tractable prior to the advent of phylogeographic approaches to assessing the relationships of distributional and phylogenetic patterns to geology and paleoclimate. We recognize that isolation and divergence between regional desert biotas is likely the result of multiple events over several time frames, including the Pleistocene, but debate continues over the importance of Late Neogene events to current North American biotic diversification and structure (e.g., Johnson & Cicero 2004). While our interpretations about _B. punctatus_ are based on a single mtDNA gene, the available data indicate that this species has three divergent maternal lineages resulting from early vicariant events. The three-way split (polytomy) between the Peninsular and continental clades implies a near-simultaneous set of vicariant events. Peninsular populations of _B. punctatus_ appear to have been separated from the mainland by either a Pliocene northern gulf vicariance or a latest Miocene–Early Pliocene southern gulf vicariance. Populations in the Sonoran and Mojave deserts were likely separated from those in the Chihuahuan Desert by the uplifting of the Colorado and
Mexican Plateaus and the Sierra Madre Occidental. These inferences are supported by the observations that where major clades have dispersed across historical barriers and are now found in sympathy, the patterns are ones of secondary contact resulting from recent population expansions. Furthermore, the deep splits between maternal lineages of *B. punctatus* among the regional deserts are generally congruent with phylogenetic subdivisions within an array of co-distributed warm desert taxa (see Riddle & Hafner *in press*) that implicated Late Neogene vicariant events as causal factors.


Riddle, B. R. 1995. Molecular biogeography in the pocket mice (Perognathus and Chaetodipus) and grasshopper mice (Onychomys): the Late Cenozoic development of a North American aridlands rodent guild. Journal of Mammalogy 76:283-301.


Fig. 2.1. Sample sites for *Bufo punctatus* in relationship to North American regional warm deserts and the Colorado Plateau (shaded areas; modified from ecoregions described by Ricketts et al. 1999). The heavy gray line indicates the approximate distributional limit of the species (after Stebbins 1985). Circle shadings indicate the
major mtDNA clades identified in the maximum likelihood tree (bottom of the figure) and in Fig. 2.2. Locations 4, 5, and 42 contain both Western and Eastern clade haplotypes. Circle size reflects sample size at each location (small circles = 1, mid-sized circles = 2-3, large circles = 4-5). Numbers identify sample localities listed in Appendix and referenced in Fig. 2.2. The maximum likelihood tree is outgroup rooted (distances to outgroups not shown).
Fig. 2.2. Bayesian inference cladogram of *Bufo punctatus* haplotypes derived from 666 bp of cytochrome b sequence data. The three major clades are discussed in text and
referenced in Fig. 2.1. There was weak support from maximum parsimony analysis for
the grouping of Peninsular and Western clades with a more basal branch leading to the
Eastern clade (indicated by grey lines). Supports for clades (> 50%) are shown with
numbers prior to forward-slash indicating Bayesian posterior probabilities (consensus of
30,000 sampled trees from three $1 \times 10^6$ generations runs) and numbers following the
slash indicating maximum parsimony bootstrap scores (1000 repetitions). The tree is
outgroup-rooted on *B. debilis* and *B. retriformis*. Unique haplotypes are identified by
letter and number designations. Numbers in parenthesis following haplotype designations
indicate sample locations referenced in Fig. 2.1. Numbers following parenthesis indicated
sample sizes greater than one. Sample and locality descriptions are listed in the
Appendix.
Fig. 2.3. (A) Nested haplotype network for mtDNA sequences of Eastern clade *Bufo punctatus* constructed under a criterion of statistical parsimony. Haplotypes are identified by letter and number designations. Numbers in parenthesis following haplotype designations indicate sample sizes greater than one. Within the network, each line between haplotypes represents a mutational change. Zeros indicate unsampled haplotypes inferred from the data. Circles surround internal haplotypes with larger sample sizes. Boxes and line-thickness indicate hierarchical nesting, with hierarchical clades designated by two-digit labels. (B) Distribution of salient lower-order clades from the nested-clade analysis of Eastern clade *B. punctatus*. Pie-graph size reflects sample size at each location progressing from smallest (n = 1) to largest (n = 5).
Fig. 2.4. Summary flow chart of nested-clade analyses results for *Bufo punctatus* haplotypes from the (A) Eastern and (B) Western clades. Nesting level increases from left to right. Numbers indicate hierarchical clades. Clade distance (Dc), nested clade distance (Dn), and interior versus tip contrast (I-T) values are indicated with those showing significantly \( P \leq 0.05 \) small or large values in bold and designated by a S or L, respectively. Shading indicates interior haplotypes or clades. Inference chain (IC) and interpretations of statistical results with abbreviations: CRE (contiguous range expansion), RE (range expansion), LDD (long distance dispersal), PF (past fragmentation), and INC (inconclusive result). Asterisks indicate inferences for clades lacking support from initial contingency tests.
Fig. 2.5. (A) Nested haplotype network for mtDNA sequences of Western clade *Bufo punctatus* constructed under a criterion of statistical parsimony. (B) Distribution of salient lower-order clades from the nested-clade analysis of Western clade *B. punctatus*. See Fig. 2.3 legend for figure explanations.
Fig. 2.6. (A) Nested haplotype network for mtDNA sequences of Peninsular clade *Bufo punctatus* constructed under a criterion of statistical parsimony. (B) Distribution of salient lower-order clades from the nested-clade analysis of Peninsular clade *B. punctatus*. See Fig. 2.3 legend for figure explanations.
Table 2.1. Representative co-distributed sister phylogroups providing evidence of (A) pre-Pleistocene Peninsular vs. western Continental deserts (Mojave and/or Sonoran desert) vicariance, or (B) pre-Pleistocene western vs. eastern (Chihuahuan) Continental deserts vicariance. Distributions across multiple deserts are indicated for some taxa.

<table>
<thead>
<tr>
<th>Co-distributed Sister Phylogroups By Desert Region</th>
<th>Western Deserts</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Peninsular Desert</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chaetodipus rufinoris</td>
<td>C. baileyi</td>
<td>7; 8</td>
</tr>
<tr>
<td>Centruroides exilicauda (Peninsular clade)</td>
<td>C. exilicauda (Continental clade)</td>
<td>2</td>
</tr>
<tr>
<td>Lophocerus schottii (Peninsular clade)</td>
<td>L. schottii (Continental clade)</td>
<td>3</td>
</tr>
<tr>
<td>Neotoma lepida (Peninsular clade)</td>
<td>N. lepida + N. devia (Continental clade)</td>
<td>7</td>
</tr>
<tr>
<td>Peromyscus fraterculus + P. eva</td>
<td>P. eremicus + P. merriami (+ Eastern Desert)</td>
<td>7; 9</td>
</tr>
<tr>
<td>Polioptila californica</td>
<td>P. melanura (+ Eastern Desert)</td>
<td>12</td>
</tr>
<tr>
<td>Sauromalus obesus (Peninsular clade)</td>
<td>S. obesus (Continental clade)</td>
<td>4</td>
</tr>
<tr>
<td>B Western Deserts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammospermophilus leucurus (+ Peninsular Desert)</td>
<td>A. interpres</td>
<td>7; 14</td>
</tr>
<tr>
<td>Callipepla douglasii</td>
<td>C. squamata</td>
<td>12; 13</td>
</tr>
<tr>
<td>Chaetodipus penicillatus</td>
<td>C. eremicus</td>
<td>1; 8</td>
</tr>
<tr>
<td>Chaetodipus artus + C. goldmani</td>
<td>C. nelsoni</td>
<td>8; 14</td>
</tr>
<tr>
<td>Kinosternon arizonense</td>
<td>K. durangoense</td>
<td>10</td>
</tr>
<tr>
<td>Onychomys torridus</td>
<td>O. arenicola</td>
<td>5; 6</td>
</tr>
<tr>
<td>Uta stansburiana (+ Peninsular Desert)</td>
<td>U. stansburiana stejnegeri</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 2.2. Measures of haplotype and nucleotide diversity of *Bufo punctatus* mtDNA cytochrome b sequence data within major clades and specific geographic regions discussed in text. The northeast Sonoran Desert includes some areas identified in Fig. 2.1 as Chihuahuan Desert habitat.

<table>
<thead>
<tr>
<th>Clade or Region</th>
<th>No. Samples (No.)</th>
<th>Haplotype Diversity ± SD</th>
<th>Nucleotide Diversity ± SD</th>
<th>Fu's $F_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eastern Clade</td>
<td>79 (19)</td>
<td>0.7147 ± 0.0531</td>
<td>0.0045 ± 0.0026</td>
<td>-5.969 ($P = 0.02$)*</td>
</tr>
<tr>
<td>Western Colorado Plateau</td>
<td>29 (4)</td>
<td>0.1995 ± 0.0977</td>
<td>0.0003 ± 0.0004</td>
<td>-3.324 ($P &lt; 0.01$)*</td>
</tr>
<tr>
<td>Chihuahuan Desert</td>
<td>50 (16)</td>
<td>0.8661 ± 0.0320</td>
<td>0.0060 ± 0.0034</td>
<td>-3.181 ($P = 0.13$)</td>
</tr>
<tr>
<td>Western Clade</td>
<td>91 (19)</td>
<td>0.8635 ± 0.0209</td>
<td>0.0067 ± 0.0037</td>
<td>-2.374 ($P = 0.24$)</td>
</tr>
<tr>
<td>Mojave Desert</td>
<td>49 (11)</td>
<td>0.8053 ± 0.0369</td>
<td>0.0061 ± 0.0034</td>
<td>0.186 ($P = 0.58$)</td>
</tr>
<tr>
<td>NE Sonoran Desert South of Gila River</td>
<td>15 (4)</td>
<td>0.3714 ± 0.1532</td>
<td>0.0006 ± 0.0007</td>
<td>-2.369 ($P &lt; 0.01$)*</td>
</tr>
<tr>
<td>Peninsular Clade</td>
<td>22 (11)</td>
<td>0.8182 ± 0.0740</td>
<td>0.0033 ± 0.0021</td>
<td>-5.114 ($P &lt; 0.01$)*</td>
</tr>
</tbody>
</table>

* Significant values
Appendix. Descriptions of sample locations, haplotypes, and sample identifications, by country, state, county, and map number (referenced in Fig. 2.1). Latitude and longitude (negative number) follow in parentheses, after location descriptions. Sample identification numbers (LVT) follow after haplotype identifications. Sequences are referenced in Genbank under accession numbers: AY010121-AY010166 and DQ085629-DQ085776.

United States: Arizona: Cochise County: 43-Paradise Cemetery, Chiricahua Mountains (31.923, 109.205), E08 (LVT4432); Coconino County: 65-Roden Spring, SW. of Roden Crater (35.416, 111.288), E01 (LVT6051); 66-Little Colorado River, at confl. with Colorado River (36.192, 111.801), E01 (LVT4480, 4483, 4486); 67-Page Golf Course, Page (36.920, 111.470), E01 (LVT4417-4419); Graham County: 40-SR266, 7 mi. SW. of jct. with US191, Pinaleno Mountains (32.559, 109.782), W05 (LVT6072); La Paz County: 31-Bill Williams River National Wildlife Refuge (34.267, 114.033), W05 (LVT5565-5566), W12 (LVT5567); Maricopa County: 32-Wickenburg (33.975, 112.733), W05 (LVT4445); Mojave County: 1-Mormon Wells, Beaver Dam Wash, (36.975, 113.985) W13 (LVT5570); 2-Cedar Break, Virgin River Gorge (36.949, 113.792), W03 (LVT5616-5619), W15 (LVT5620); 30-Kaiser Spring Wash, 10 mi. S. of Wikieup (34.586, 113.495) W05 (LVT5621); Navajo County: 64-Clear Creek near McHood Park Lake (34.968, 110.644), E01 (LVT5622-5626); Pinal County: 37-Buzzard Roost Spring, Galiuro Mountains (32.786, 110.547), W05 (LVT3573-3575); Pima County: 39-Arivaca Road, 2.2 mi. NW. of Arivaca (31.595, 111.316), W07 (LVT4446); Santa Cruz County: 38-Mt. Hopkins Road, Santa Rita Mountains (31.693, 110.957), W05 (LVT6070), W16 (LVT6071); Yavapai County: 33-Humbug Creek, N. of Lake Pleasant (33.975, 112.291), W05 (LVT4577), W06 (LVT4576); Yuma County: 34-High Tank 8, Kofa Mountains (33.379, 114.031), W05 (LVT5583-5585); 35-Palm Road, Kofa Mountains (33.337, 114.147), W12 (LVT6060, 6062); California: Inyo County: 23-Great Falls Basin, Argus Range (35.855, 117.381), W14 (LVT5592-5596); Riverside County: 24-Rawson Canyon (33.617, 117.025), W08 (LVT4596); 28-Cottonwood Spring, Eagle Mountains (33.738, 115.810), W05 (LVT5589, 5591), W12 (LVT5590); 29-Canyon Spring, Oroopia Mountains (33.546, 115.655), W05 (LVT5588); San Bernardino County: 13-Horse Thief Springs, Kingston Range (35.773, 115.886), W01 (LVT3478-3480); 14-Colosseum Gorge Spring, Clark Mountain (35.554, 115.554), W01 (LVT4256); 19-Fort Piute, Piute Range (35.112, 114.997), W10 (LVT4459); 20-Vernandless Spring, Marble Mountains (34.687, 115.642), W01 (LVT3507-3508, 4407); 21-Dripping Spring, Old Women Mountains (34.560, 115.209), W06 (LVT5598, 5599), W13 (LVT5600); 22-Horn Spring, Turtle Mountains (34.208, 114.788), W05 (LVT5587), W06 (LVT5586); San Diego County: 25-Borrego Palm Canyon, Anza Borrego Desert State Park (33.278, 116.428), W08 (LVT4448); 26-Auga Caliente
Springs, Anza Borrego Desert State Park (32.948, 116.305), W06 (LVT4474, 4476), W08 (LVT4473), W11 (LVT4475, 4477); 27-Dos Cabezas Spring, Anza Borrego Desert State Park (32.715, 116.143), W08 (LVT4478), W11 (LVT4479); Nevada: Clark County: 3-Magnesite Wash, S. of Overton (36.501, 114.471), W03 (LVT2762); 4-Red Bluff Spring, Gold Butte area (36.464, 114.256), E01 (LVT2825-2826), W03 (LVT2824); 5-Surpus Spring, E. of Muddy Mountains (36.377, 114.445), E01 (LVT2777), W02 (LVT2776), W03 (LVT2778); 6-Cottonwood Spring, Black Mountains (36.204, 114.476), W03 (LVT4147-4149); 7-Connoly Spring, Gold Butte area (36.246, 114.108), W03 (LVT2990-2902); 8-Pupfish Refugium Spring, Black Canyon (36.009, 114.747), W02 (LVT2959), W03 (LVT2958), W04 (LVT2960); 9-Kiup Spring, Spring Mountains (36.163, 115.722), W01 (LVT4311); 10-La Madre Spring, Spring Mountains (36.187, 115.506), W01 (LVT3022-3023), W05 (LVT3024); 11-Pine Creek, Spring Mountains (36.122, 115.470), W01 (LVT2666); 12-Lost Cabin Spring, Spring Mountains (36.085, 115.650), W03 (LVT4236); 15-McCullough Spring, McCullough Range (35.649, 115.128), W03 (LVT3251); 16-Railroad Spring, McCullough Range (35.604, 115.198), W01 (LVT4287, 4291); 17-Highland Spring, Highland Range (35.597, 115.058), W06 (LVT3290); 18-Cow Spring, Highland Range (35.569, 115.014), W01 (LVT3313); New Mexico: Dona Ana County: 48-Corrallitos Ranch Road, 19.4 mi. NW. of jct. with I-10 (32.500, 107.100), E03 (LVT6073), E07 (LVT6074-6075); Eddy County: 49-SR137, 12.3 mi. S. of US285 (32.453, 104.483), E01 (LVT4436); 50-SR137, 12.6 mi. N. of TX State Line (32.158, 104.792), E03 (LVT4435, 4437); Grant County: 44-Evan's Lake, (32.868, 108.580), E01 (LVT4449); 45-Martain Canyon, 1.5 mi. N. & 4.2 mi. E. of Hurley (32.717, 108.075), E01 (LVT4453, 4455), E09 (LVT4454); Guadalupe County: 61-SR91, 0.6-3.6 road mi. N. of Pueta de Luna (34.835, 104.619), E01 (LVT5629), E13 (LVT5627, 6054), E14 (LVT5628); Hidalgo County: 41-Box Canyon, SE. of Verden Bridge (32.628, 108.857), W05 (LVT4451-4452), W09 (LVT4450); 42-Granite Gap, Peloncillo Mountains (32.089, 108.974), E07 (LVT4426), W05 (LVT4427-4430); Luna County: 46-SR9, 26.2 mi. W. of Columbus (31.813, 108.048), E03 (LVT3416); 47-SR9, 19.2 mi. W. of Columbus (31.848, 107.944), E02 (LVT3414); San Juan County: 69-US64, 4.4 mi. E. of AZ state Line (36.828, 109.001), E01 (LVT4441); San Miguel County: 60-SR104, between Cochis and Tucumcari, 12 mi. N. of county line (35.315, 103.972), E01 (LVT5631, 5634-5635), E13 (LVT5632), E15 (LVT5633); Socorro County: 63-Carbon Spring, NE. of Magdalena (34.321, 107.195), E01 (LVT4401); Torrance County: 62-US60, 2.5 mi. E. of Socorro County Line (34.439, 106.380), E01 (LVT4438), E03 (LVT4439-4440), E09 (LVT5641-5642); Texas: Brewster County: 53-Road from Persimmon Gap, Big Bend National Park (29.517, 103.123), E01 (LVT4412, 4415), E03 (LVT4414, 4416), E06 (LVT4413); 54-Rio Grande Village, Big Bend National Park (29.192, 102.950), E10 (LVT4595); Gillespie County: 56-On loop road, 2.35 mi. N. of Willow City (30.448, 98.655), E01 (LVT4404, 4406), E04 (LVT4405); Hall County: 58-SR70, 9.8 mi. N. of Turkey (34.528, 100.929), E13 (LVT6056-6067, 6059), E17 (LVT6058); Jeff Davis County: 51-FM1832, 10.6 mi. W. of SR17 (30.808, 103.910), E01 (LVT6076, 6078), E19 (LVT6077); Motley County: 57-SR70, 2.1 mi. S. of Matador (33.982, 100.829), E13 (LVT6055); Pecos County: 52-FM2886, 14.5 mi. S. of I10 (30.708, 102.193), E18 (LVT6069); Randall County: 59-Rim of Palo Duro Canyon (34.933, 101.140), E16 (LVT5636-5638); Val Verde County: 55-Comstock, Jct.US90
and SR163 (29.688, 101.173), E18 (LVT6068); Utah: Garfield County: 72-Hog Spring, SE. of jct. SR95 and SR276 (37.969, 110.511), E01 (LVT5607-5609), E11 (LVT5606), E12 (LVT5610); Kane County: 68-Wahweap State Fish Hatchery, Big Water (37.092, 111.667), E01 (LVT4422-4425), E05 (LVT4411); San Juan County: 70-Visitor center, Hovenweep National Monument (37.390, 109.082), E01 (LVT6053); 71-Hovenweep Road, 0.2 mi. W. of state line (37.423, 109.048), E01(LVT6052); Mexico: Baja California: 73-Catavina (29.733, 114.717), P03 (LVT1789), P04 (LVT1785, 1787-1788), P05 (LVT1786); 74-Bahia de Los Angeles (29.019, 113.800), P04 (LVT4468), P10 (LVT4469); 75-Rosorito (southern city) (28.516, 114.030), P03 (LVT4470); Baja California Sur: 76-San Francisco de la Sierra (27.583, 113.033), P01 (LVT1780), P02 (LVT1781), P03 (LVT1782-1784); 77-Santa Agueda (27.259, 112.350), P03 (LVT4471-4472); 78-San Pedro (23.790, 110.130), P08 (LVT4465); 79-Auga Calienta (23.442, 109.803) P03 (LVT4460), P06 (LVT4462), P07 (LVT4463), P11 (LVT4461); 80-Boca de La Sierra (23.388, 109.816), P03 (LVT4467), P09 (LVT4466); Sinaloa: 82-Hwy, 32 N. of Hiox (26.833, 108.370), W17 (LVT6358), W18 (LVT6359); Sonora: 36-Sonoita (Soroita) (31.850, 112.850), W05 (LVT4464); 81-Between Hornos and San Nicolas (27.776, 109.537), W19 (LVT6360).
CHAPTER III

GENETIC STRUCTURE OF THE RED-SPOTTED TOAD, *BUFO PUNCTATUS*,
IN A NATURALLY FRAGMENTED DESERT LANDSCAPE

Abstract

We provide insight on the spatial scale at which the genetic structure of *Bufo punctatus* within the Mojave Desert is organized, and evaluate two alternative hypotheses for population structure, nonequilibrium populations and patchy populations. A portion of mitochondrial DNA control region was sequenced for 831 toads collected from 43 sites around Las Vegas, Nevada. Collection sites were grouped *a priori* into seven geographic ranges, based predominately on clusters of breeding sites within mountain range groups. Major lineages were identified by network analysis. Neutrality test statistics were used to infer mountain range-wide population bottlenecks, expansions, or past subdivision. Hierarchical analysis of molecular variance (AMOVA) was used in a series of nested procedures to assess genetic structure among mountain ranges, among sites within mountain ranges, and among individuals within sites. Pairwise $F_{ST}$ were calculated among sites within mountain ranges to further elucidate substructure. Thirty-six haplotypes within 5 major lineages were identified. An additional haplotype representative of *B. punctatus* recently expanding from the Colorado Plateau and
Chihuahuan Desert was also found at three sites. The study-wide AMOVA indicated that a significant amount (25.8%) of genetic variation was accounted for by the designated mountain ranges. Substantial genetic structure between most neighboring mountain ranges was confirmed by pairwise AMOVAs. Within 4 of the 7 mountain ranges, little to no genetic variation was determined among breeding sites. A population bottleneck or range expansion was inferred to explain the lack of haplotypic diversity within two of these mountain ranges. Within those mountain ranges showing significant genetic structure among sites, pairwise $F_{ST}$ comparisons indicated that only a few sites were generally responsible for the observed structure. Recent convergence of two divergent lineages appears to have caused most of the genetic structure within one range. Our analysis supports the perspective that *B. punctatus* within the Mojave Desert occurs primarily in patchy populations within mountain ranges that are isolated from patchy populations in other mountain ranges.

Introduction

Within the extremely dry climate of the Mojave Desert, the local distribution of *Bufo punctatus* Baird and Girard 1852, the red-spotted toad, appears to coincide, at least superficially, with the fragmentation of its aquatic breeding habitat. This warm-desert toad depends predominately on rain catchments, springs and associated small streams for breeding (Sullivan 2005). In the Mojave Desert, it avoids dense vegetation and is positively associated with rocky habitats, ephemeral waters, and sites that are periodically scoured by floods (Bradford et al. 2003). Wetland sites with these habitat characteristics occur mostly along the lower slopes and canyons of desert mountain ranges, separated by
expanses of arid terrain or by higher elevation mountain ridges not associated with this species.

Like many bufonids, *B. punctatus* has physiological and behavioral traits that allow substantial forays into arid terrain (Shoemaker 1988; Shoemaker et al. 1992). Despite these traits, only limited dispersal of up to about 0.8 km has been observed (Turner 1959; Tevis 1966; Weintraub 1974; McClanahan et al. 1994). The scattered distribution of wetland sites within the Mojave Desert and the presumed limited vagility of *B. punctatus* led some authors to speculate that its distribution was originally established during more mesic climatic conditions (Storer 1925, in Turner 1959). Populations within the climatically extreme Death Valley region (i.e., northern Mojave Desert) were assumed, "...to be genetically isolated colonies derived from a once more extensive Pleistocene distribution..." (Turner 1959, p. 180). Bradford et al. (2003), however, reasoned that the paleoclimate within the northeastern fringe of the Mojave Desert was too cold and mesic during the last glacial period for *B. punctatus*. These authors speculated that *B. punctatus* may have expanded into the region at the end of the Pleistocene from lower elevation corridors, or refugia, along the Colorado and Amargosa river drainages (the latter drains into the Death Valley region). In particular, the lower Colorado River, extending northward into eastern Mojave Desert, appears to have maintained warm desert conditions during the height of the last glacial period (Bentancourt et al. 1990; Thompson and Anderson 2000). Consistent with both these scenarios are high measures of mitochondrial DNA (mtDNA) diversity observed within *B. punctatus* samples from the Mojave Desert that indicate populations were likely to
have persisted within at least portions of this region during the Pleistocene (Jaeger et al. 2005).

From an ecological perspective, fragmented breeding habitats, patchy distributions, and limited organism vagility describe conditions consistent with classical metapopulation theory (sensu Levins 1969) as an explanation for current population distributions (see Hanski and Gilpin 1991). Amphibians have often been assumed to exist in metapopulations; however, reviews of the evidence have raised questions about the metapopulation paradigm (Marsh and Trenham 2001; Smith and Green 2005). Findings from a recent meta-analysis indicate that amphibian dispersal appears to have been commonly underestimated and movements may not be uniformly limited across species (Smith and Green 2005). These observations indicate that for many anurans migration between breeding habitats may be much higher than commonly assumed.

In a study of habitat patch occupancy by *B. punctatus*, Bradford et al. (2003) tested two hypotheses concerning habitat patch size and isolation that are predicted for a metapopulation at equilibrium (Hanski 1994, 1998, 1999). These authors determined that patch occupancy by *B. punctatus* within the northeastern Mojave Desert was related to patch size as predicted, but not related to patch isolation as predicted. While these observations did not fit a classical metapopulation model, the data were consistent with two alternative models, nonequilibrium and patchy populations (see Harrison 1991; Harrison and Taylor 1997). Nonequilibrium populations differ from a classical metapopulation in that migration between local populations is so limited that recolonization of habitat sites after local extinction will not occur (on an ecological time scale). A patchy population differs from a classical metapopulation in that migration
rates among local habitat patches are so high that demographic dynamics are not independent and local extinction is uncommon.

Bradford et al. (2003) reasoned that the nonequilibrium model was less likely for *B. punctatus* because of the discord between high patch occupancy rate and apparent small population sizes within habitat patches (thus assuming that isolated populations would have high extinction probabilities). They concluded that dispersal was likely high among sites and that *B. punctatus* distribution was best described by a patchy population model. Their analytical approach, however, did not account for inter-mountain range patterns so they could not determine a spatial scale at which the patchy populations might have been structured. The conclusion of patchy populations was perplexing to these authors because it required either that they greatly underestimated the number of habitat patches occupied by *B. punctatus* or that the vagility of this toad was greatly underestimated from previous studies. The latter explanation fits the expectation that anuran dispersal has generally been underestimated (Smith and Green 2005).

Herein, we present research that followed the analysis of Bradford et al. (2003) and assessed local patterns of population structure and dispersal of *B. punctatus* using mtDNA control region sequence data. Our sampling focused on breeding sites, and assessments were based at two geographic scales, among breeding sites clustered within local ranges (generally within mountain ranges), and among the groupings of sites (generally among mountain ranges). The main objectives of this study were to provide insight on the spatial scale of genetic structure and to evaluate the two alternative models, nonequilibrium and patchy, for the population structure of *B. punctatus* within the northeastern Mojave Desert. These two models defined opposite ends of a continuum of
possible population structures that can exist within a fragmented landscape. In *B. punctatus*, these population structures could be manifested at different geographic scales, with the observation of nonequilibrium or patchy structure among mountain ranges potentially coupled with either nonequilibrium or patchy population structure among sites within each mountain range.

The genetic consequences for the two population models are straightforward (e.g., Harrison and Hastings 1996). For the nonequilibrium model, we expected to observe random loss of mtDNA diversity within isolated populations resulting in high measures of genetic divergence (structure) among areas with migration rates significantly less than one individual per generation (Hartl and Clark 1997). Alternatively, under a patchy population model, high levels of migration among areas would restrict genetic drift and result in little genetic structure. Assuming sufficient time, the genetic differences between these alternative population structures should be apparent using hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) and other measures of genetic structure (e.g., Wright's $F_{ST}$ statistic).

Material and Methods

Study Area

Approximately 20,000 km$^2$ of the northeastern portion of the Mojave Desert was delineated for study (Fig. 3.1). This area was centered on Las Vegas Valley in southern Nevada and extended into areas of northwestern Arizona and southeastern California. The region encompasses numerous desert mountain ranges and valleys, with roughly the eastern two-thirds draining to the lower Colorado River system (i.e., Colorado, Virgin,
and Muddy Rivers) and the western third draining into several enclosed basins including the mostly ephemeral Amargosa River system. The area was delineated to include populations of *B. punctatus* near and remote from the lower Colorado River which was thought to be the likely source of post-glacial population expansions for this species within the study area (Bradford et al. 2003).

Sampling and Hierarchical Grouping

Nearly all springs and large rain catchments within acceptable elevation limits for *B. punctatus* were surveyed for the presence of this toad (n = 128 sites). Details for these surveys were previously described in Bradford et al. (2003), although we included an additional site (Fig. 3.1, Overton Arm site 8), and lumped two closely situated sites (Fig. 3.1, Spring Range site 2). Where *B. punctatus* was found (n = 93 sites), tissue samples were collected, predominately toes from adult animals captured and released. For population genetic evaluation, sites were limited to those where sufficient numbers of individuals (10-25) were sampled. A total of 43 sites (Fig. 3.1) incorporating 831 samples were included for analyses.

Sites were grouped *a priori* into seven geographic ranges (Fig. 3.1). We used geographic ranges based predominately on natural clusters of springs within mountain range groups previously defined within the study area by Bradford et al. (2003) except that sites along the Colorado River system were split into two ranges, those within Black Canyon (along the Colorado River below Lake Mead in both the Eldorado and Black Mountains) and those around the Overton Arm of Lake Mead (springs within the Black and Muddy Mountains along the former Virgin River just above its confluence with the Colorado River, and along the Muddy River). These latter two geographic ranges both
defined clusters of sites bordering the river system, but sites within Black Canyon formed a distinct cluster separate from sites around the Overton Arm, especially since we were unable to get sufficient samples from several intervening springs. Because most of these geographic regions consisted of specific mountain ranges, heretofore, we refer these units simply as ‘mountain ranges’ or by the names referenced in Fig. 3.1.

Laboratory Methods

Laboratory protocols were almost identical to those described by Jaeger et al. (2005). In general, total genomic DNA was extracted from toe tissue, or occasionally muscle, using a standard phenol-chloroform technique. A portion of the mtDNA control region comprising the end of cytochrome b (cyt b), the variable left domain, and the conserved central domain was amplified by polymerase chain reaction (PCR) using primers CytbA-L and ControlP-H (Goebel et al. 1999). Fluorescence-based cycle sequencing was then conducted on the PCR templates. Electrophoresis and visualization of sequences were completed on an ABI 377 automated sequencer or occasionally on an ABI 310 automated sequencer (PE Applied Biosystems, Inc.). In a preliminary evaluation of genetic variation, the majority of the PCR template was sequenced for single individuals from most of the collection sites (data not shown). From this preliminary analysis, a 585 basepair segment, following a variable repeat region at the beginning of the control region, was selected for further analyses. A slightly modified version of primer ControlB-H (Goebel et al. 1999) was used to sequence this targeted region.
Phylogenetic Methods

Sequence alignments were made by eye using BioEdit (version 5.0.9; Hall 1999). Haplotypes were identified by calculating the number of nucleotide differences among sequences using pairwise deletion of ambiguous sites (maximum ambiguity of any sequence was < 0.019). A haplotype network (Posada and Crandall 2001) was derived under a parsimony algorithm (Templeton et al. 1992) and 95% probability criterion for connections using the program TCS (version 1.6, Clement et al. 2000). The major lineages identified in the network were confirmed by conducting a tree-based maximum parsimony (MP) analysis on a subset of the data in which identical haplotypes were merged. Samples from the Eastern clade and Peninsular clade of *B. punctatus* (Riddle et al. 2000; Jaeger et al. 2005) were used as outgroups. Unweighted MP analysis was implemented in MEGA2 (Kumar et al. 2001) by employing a heuristic search algorithm (close-neighbor-interchange branch swapping with search level 2, random stepwise addition, 100 replications, all sites option). Resulting MP topologies were assessed for robustness using nonparametric bootstrap (500 replicates) under the same search criteria (but with random stepwise addition reduced to 10 replications). Estimates of net sequence divergence among the clades, corrected for variation among haplotypes within each of the clades, were calculated as p-distances (pairwise deletion) in MEGA2.

Neutrality Test Statistics

To evaluate departures from expectations of neutral equilibrium dynamics in the genetic structure of populations, Tajima’s *D* statistic (Tajima 1989) and Fu’s *F*_s test (Fu 1997) were calculated within ARLEQUIN (Schneider et al. 2000). In the absence of selection, strongly negative values for these statistics are thought to be evidence of
population bottleneck or expansion (Tajima 1989; Simonsen et al. 1995; Fu 1997). Conversely, populations with a history of past subdivision are expected to show positive $D$ statistics (Simonsen et al. 1995). For these analyses, samples from sites within each mountain range were pooled. Critical values for the $D$ statistics were determined assuming the beta-distribution as implemented in ARLEQUIN (Schneider et al. 2000). For the $F_S$ tests, $P \leq 0.02$ were assumed to be significant (Schneider et al. 2000).

Hierarchical Analysis of Molecular Variance

To assess genetic structure at various levels of geographic organization, we used hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) in a series of nested procedures. Genetic structure was assessed at three hierarchical scales: within sites, among sites within mountain ranges, and among mountain ranges (see Fig. 3.1). At the most inclusive level, an AMOVA was used to partition total genetic variance across the study area. This analysis provided an overall assessment of the importance of the designated mountain ranges and sites within ranges to genetic structure. Haplotype correlation measures, $\Phi$-statistics, were generated with the AMOVA analyses and the following relationships for these statistics are maintained throughout the paper: $\Phi_{CT} =$ among mountain ranges, $\Phi_{SC} =$ among sites within mountain ranges, and $\Phi_{IT} =$ among individuals within sites.

To further assess genetic structure among mountain ranges, a stepping-stone model among the ranges was assumed, and AMOVAs were performed on 10 pairs of ranges in which a connection was likely (i.e., mountain ranges that were adjacent to one another or were directly connected through drainage basins). These pairwise AMOVAs provided $\Phi$-statistics (pairwise $\Phi_{CT}$) between pairs of mountain ranges. At the least
inclusive level, one-mountain range AMOVAs were conducted to assess genetic patterns among sites within each individual mountain range (range specific \( \Phi_{SC} \)). For those mountain ranges demonstrating substantial among-site (within-range) genetic structure, pairwise \( F_{ST} \) were calculated among sites within each mountain range to further elucidate patterns.

AMOVAs and pairwise \( F_{ST} \) values were calculated assuming p-distances using ARLEQUIN. Significance of fixation indices was determined by 10,000 non-parametric permutations (Schneider et al. 2000). Sequential Bonferroni corrections were used to account for simultaneously conducted tests of pairwise \( F_{ST} \) values (Rice 1989).

Haplotype \( (h) \) and nucleotide diversity \( (\pi) \) were estimated (Nei 1987) using ARLEQUIN.

Results

Haplotypes

Across its broad distribution within the warm deserts of North America, \( B. \ punctatus \) is comprised of three major mtDNA lineages (i.e., Eastern, Western, and Peninsular clades; Riddle et al. 2000; Jaeger et al. 2005). Consistent with the previously defined Western clade of \( B. \ punctatus \), 36 control region sequence haplotypes were identified from the 831 individuals examined. In addition, a single haplotype (a total of 9 individuals) from the previously identified Eastern clade was found at three sites adjacent to the Overton Arm of Lake Mead (see Discussion). Western clade haplotypes differed from the Eastern clade haplotype by 0.063–0.082 (p-distances). Among the Western clade sequences, pairwise nucleotide differences ranged from 1 to 15 (p-distances ≤ 0.026). A total of 41 nucleotide sites varied, of which 25 were parsimony informative.
These values increased to 80 variable sites, with 39 parsimony informative, when outgroup sequences of *B. punctatus* from Eastern and Peninsular clades were included in the analysis.

**Phylogenetic Relationships**

Network analysis resulted in 5 major control region lineages (Fig. 3.2). These same lineages were depicted on the MP consensus tree (not shown). Herein, we refer to these lineages as ‘clades’ A through E. The network contained a loop connecting clades A, B, and C. This ambiguity was manifest on the MP consensus tree by an unresolved position of clades A and B relative to C. Clades A and B were closely related, separated by only two steps in the network. Clades C, D, E, and a combined A and B clade differed from one another by 6 or more steps within the network. Clades A and B were poorly supported in the MP analysis (BS for clade A = 62, BS for clade B < 50), but the combined A and B clade was strongly supported (BS = 91). The other major clades received moderate to high support (BS = 65–97). Net sequence divergence (p-distances) among clades (Table 3.1) ranged from 0.004 (between clades A and B) to 0.018 (between clades A and E).

The relationships between the major control region clades and previously described cyt b haplotypes and one-step nested clades identified within the study area by Jaeger et al. (2005) were evaluated by comparing individuals sequenced for both mtDNA segments (Table 3.2). Control region clades A, B, and E directly corresponded to previously identified cyt b Western clades 1-1, 1-4, and 1-10, respectively (see Fig. 5 in Jaeger et al. 2005). Individuals within clade C had cyt b sequences that matched haplotypes within Western clades 1-7 and 1-8 which were closely related within the cyt b
network. The main haplotype within clade D corresponded to a previously unidentified
cyt b haplotype closely related to Western clade 1-10.

Neutrality Test Statistics

Tajima’s $D$ and Fu’s $F_s$ statistics showed no significant positive or negative
trends for the majority of mountain ranges (i.e., Spring Mountains, Eldorado Mountains,
Black Canyon, and McCullough Range). Both the Clark Mountain and the Kingston
Range lack haplotype diversity when compared to the other mountain ranges and the
neutrality statistics were significantly negative for Clark Mountain (Table 3.3). The
neighboring Kingston Range contained only a single haplotype (thus these tests could not
be performed), but the haplotype in this range was identical to the overwhelmingly
dominant haplotype in Clark Mountain (i.e., haplotype Ad). Tests run on a pooled
dataset from these two ranges resulted in significantly negative values ($D = -1.626, P =
0.04; F_s = -5.444, P < 0.01$). Tajima’s $D$ and Fu’s $F_s$ statistics were strongly positive for
the Overton Arm range (Table 3.3). Within this range, divergent haplotypes from clades
B and C occur in roughly equivalent frequencies (Fig. 3.3).

Regional AMOVA

Significant geographic components to population structure were indicated by the
hierarchical AMOVA. Within the study area, 25.8% of mtDNA variation was accounted
for among the designated mountain ranges ($\Phi_{CT} = 0.258; P < 0.001$), while 12.6% of
genetic variation was partitioned among sites within mountain ranges ($\Phi_{SC} = 0.170; P <
0.001$). Differences among individuals within sites accounted for 61.6% of the variation
($\Phi_{IT} = 0.384; P < 0.001$).
Among-Mountain Range Patterns

In pairwise AMOVAs among mountain ranges considered to be stepping-stones (Fig. 3.3), variation was determined to be non-significant between Clark Mountain and the Kingston Range ($\Phi_{CT} < 0.017$, $P = 0.107$), and between Overton Arm and Black Canyon ($\Phi_{CT} = 0.052$, $P = 0.092$). Very low, but significant, genetic structure was determined between the Eldorado Mountains and McCullough Range ($\Phi_{CT} = 0.075$, $P = 0.003$). Conversely, substantial amounts of genetic variation were indicated between the Clark and Kingston ranges and the neighboring Spring Mountains and McCullough Range ($\Phi_{CT} > 0.329$, all $P < 0.034$). The Spring Mountains also neighbor the McCullough Range, Black Canyon, and Overton Arm, with the latter two ranges separated from the Spring Mountains by the Las Vegas Valley (Fig. 3.1). Pairwise AMOVAs between the Spring Mountains and these other mountain ranges indicated substantial levels of genetic structure ($\Phi_{CT} = 0.176–0.231$, all $P < 0.001$). A surprising level of genetic structure was also indicated between Black Canyon and the Eldorado Mountains ($\Phi_{CT} = 0.205$, $P = 0.002$), even though these two ranges are closely situated and connected by the Colorado River and its tributary drainage channels.

Within-Mountain Range Patterns

When genetic variance within each mountain range was individually evaluated to determine the component attributable to patterns among sites (one-mountain range AMOVAs), very low and predominately non-significant variation (all $\Phi_{SC} < 0.052$, Table 3.3) was determined among sites within Clark Mountain, Eldorado Mountains, and McCullough Range. The Kingston Range lacked any haplotype diversity. Sites within
Black Canyon, Overton Arm, and the Spring Range showed much higher and significant levels of among-site structure ($\Phi_{SC} = 0.125–0.288$; Table 3.3).

Within these mountain ranges showing significant structure among sites, pairwise $F_{ST}$ comparisons among sites within Black Canyon and within the Spring Mountains indicated that only a few sites were generally responsible for most of the observed population substructure within each mountain range. Within Black Canyon, sites 2 and 3 (Fig. 3.1) were fixed for a single haplotype and these two sites accounted for the majority of the divergence (significantly high pairwise $F_{ST}$ values) within this range (Table 3.4). Within the Spring Mountains, most sites were clustered on the east side of the mountain range (Fig. 3.1, Spring Mountains sites 4–11). The four sites outside this cluster generally contained fewer haplotypes than those within the cluster (about half as many when corrected for sample size differences; see Appendix). Two of these sites (Fig. 3.1, Spring Mountains sites 2 and 3) accounted for most of the significant among-sites divergence (Table 3.5). The pattern within the Overton Arm range appeared complex and may have resulted from the convergence of historically isolated clades (see Discussion). Two sites draining to the Muddy River (Fig. 3.1, Overton Arm sites 1 and 2) contribute much to the observed differences within this range (Table 3.6).

Discussion

Among-Mountain Range Patterns

At scales above that of a single mountain range, the designated mountain ranges accounted for a significant amount (25.8% in the study-wide AMOVA) of *Bufo punctatus* mtDNA variation within the northeastern Mojave Desert. In pairwise AMOVAs between
mountain ranges considered to be adjacent or connected (Fig. 3.3), substantial levels of
 genetic structure among most mountain range pairs were indicated. However, low levels
 of genetic structure were observed in 3 of the 10 comparisons. We expected lower levels
 of between-range genetic structure for those ranges closer to the Colorado River
 drainage, because we had assumed that the river was the source for post-Pleistocene
 expansion into these mountain ranges (Bradford et al. 2003) and that the river system
 would facilitate large populations and high dispersal. We were surprised, however, by
 the high $\Phi$-statistic for the pairwise comparison between Black Canyon and the Eldorado
 Mountains. We thought that the two sites within Black Canyon that contained
 populations fixed for a particular clade B haplotype may have inflated the $\Phi$-statistic in
 this comparison, but a moderate difference between the two ranges remained even after
 the exclusion of these sites ($\Phi_{CT} = 0.132, P < 0.001$).

The lack of genetic variation between (and within) Clark Mountain and the
 Kingston Range could be explained by coincidental genetic drift to fixation of haplotypes
 or by a potential range expansion. A large wash (the Kingston Wash) and a ridgeline
 (Mesquite Mountains) mostly connect these two ranges, but both these intermediate areas
 lack permanent water. Populations within these two mountain ranges appear to be small
 and isolated, but if drift was solely responsible for the observed patterns, it seems
 unlikely that both mountain ranges would be dominated by the same haplotype (i.e.,
 haplotype Ad; Fig. 3.3). A more probable explanation is a range expansion. This
 expansion may have occurred from populations in the McCullough Range, in which the
 dominate haplotype observed in the other mountain ranges also occurs, or from
 unsampled populations to the south or west (these sites share drainages to the Amargosa
River). An associated cyt b haplotype (i.e., W01; Table 3.2) was identified in one mountain range further to the south (Jaeger et al. 2005). Unfortunately, our sampling was only sufficient to infer a probable range expansion and not to address the source or scale of this expansion.

The low levels of genetic structure between some pairs of mountain ranges indicate that relatively high migration rates between ranges may be possible, but the overall strong genetic structure associated with mountain ranges implies little migration. This implication of limited migration between mountain ranges was further supported by the fact that most mountain ranges contained many private haplotypes (Fig. 3.3), with some of these private haplotypes being common within a range. If mountain ranges were generally connected by high levels of current migration we would expect few private haplotypes (Slatkin 1985). These data support the perspective that most sites within mountain ranges currently exchange few migrants with sites in other mountain ranges.

Within-Mountain Range Patterns

A moderate level of genetic variation among B. punctatus from the northeastern Mojave Desert was associated with patterns among breeding sites within mountain ranges (12.6% in the study-wide AMOVA). Only a few mountain ranges, however, appeared to manifest much of this intra-range variation. When genetic variation within each mountain range was evaluated independently (using single-range AMOVAs), little to no genetic variation among sites was determined for 4 of the 7 mountain ranges. Even within those mountain ranges showing significant genetic structure among sites, pairwise $F_{ST}$ comparisons indicated that only a few sites within each mountain range were generally responsible for the observed structure. Several of the most divergent sites were
fixed for a particular haplotype which strongly influences \( F_{ST} \) measures (Charlesworth 1998).

In general, our observations indicated only limited structuring of haplotypes among the majority of breeding sites within mountain ranges. Although this pattern could have resulted from insufficient time for sorting of ancestral haplotypic diversity within recently isolated populations (Slatkin and Maddison 1989), our observations lead us to interpret this low amount of genetic structure as consistent with the expectation of limited divergence among populations caused by relatively high migration. Smith and Green (2005) reviewed information on anuran movements and predicted that, in general, only when habitat patch networks are separated by > 10 km will migration rates be low enough (i.e., \( Nm << 1 \)) for population differentiation. This general expectation was derived across multiple species, and these authors cautioned that the patterns observed in any particular situation will depend on the actual dispersal ability of the species and the environmental conditions between habitat patches. Numerous studies have noted that genetic structure in anurans appears to be sensitive to the habitat conditions that separate populations (Hitchings and Beebee 1997; Rowe et al. 2000; Lampert et al. 2003; Funk et al. 2005). High levels of genetic differentiation between anuran populations at scales < 10 km have been documented (Driscoll 1998; Rowe et al. 2000; Shaffer et al. 2000), but in general, very low levels of genetic structure (thus high migration rates) appear common among populations at spatial extents of similar magnitudes (see Table 3.3 in Monsen and Blouin 2004). Within the northeastern Mojave Desert, nearest neighbor distances among sites occupied by *B. punctatus* were predominately < 10 km (Euclidian
distances; Bradford et al. 2003) which was consistent with the general observation of limited within-range genetic divergence among sites.

Within those mountain ranges demonstrating significant genetic structure among sites, the causal factors resulting in the distinctiveness of populations at certain sites appear to be idiosyncratic. Isolation (by distance or by non-traversable mountain ridges) appears to be responsible for some genetic variation, particularly within the Spring Mountains. However, even in high migration systems, declines or temporary losses in local populations can result in bottlenecks or extinction and recolonization dynamics that produce transient differentiation through founder events (McCauley 1993). Many wetland sites occupied by *B. punctatus* within the Mojave Desert are quite small, with water extending a median distance of only 200 m and median area of 72 m² (Bradford et al. 2003). Presumably, many of these sites are susceptible to extended droughts or other stochastic events. With sufficiently high local dynamics, at least some sites may appear distinctive at any point in time.

The observed lack of haplotype diversity within closely situated sites within Black Canyon, as well as the dominance of two closely situated sites within the Spring Mountains by different haplotypes (Fig. 3.1, Spring Range sites 2 and 3), implicate population bottlenecks or founder events as potential causal factors. For example, the two sites within Black Canyon that were fixed for the same common haplotype occupy narrow canyons exposed to recurrent flash floods that often occur at times when toads are active. We suspect that portions of these populations may be intermittently washed into the Colorado River (in this area, the Colorado River currently emerges from the bottom of Hoover Dam and Lake Mead, and the cold, controlled flow produces a system not
generally occupied by *B. punctatus*). Our samples from these sites come from the lower ends of these narrow canyons, and the observed lack of haplotypic diversity may have resulted from local founding events following relatively recent flash floods.

Introgression of two historically isolated haplotypic clades appears to be responsible for some of the genetic structure within the Overton Arm range. The possibility of this nonequilibrium situation was implicated by the strongly positive neutrality test statistics (Table 3.3). Within this range, divergent haplotypes from clades B and C occur at roughly equivalent frequencies (Fig. 3.3). Clade B is comprised of a diverse assemblage of 7 haplotypes (gene diversity = 0.787 ± 0.019) while clade C is comprised of only 4 haplotypes (gene diversity = 0.280 ± 0.077) dominated by a single haplotype (84% of clade C samples are haplotype Ch). High percentages of clade C haplotypes occur at the sites with more direct drainage connections to Lake Mead. A possible explanation for this pattern is that clades B and C recently converged within this range, with clade B having occupied the area prior to an invasion by haplotypes from clade C, likely from areas down-river. Several of the lake-side populations within this range (Fig. 3.1, Overton Arm sites 4, 5, and 8) also contained low frequencies of a Eastern clade haplotype (not included in comparisons). Phylogeographic analyses indicate that Eastern clade *B. punctatus* colonized the northeastern Mojave Desert from the Colorado Plateau and Chihuahuan Desert during the Holocene, likely from upstream locations along the Colorado River (Jaeger et al. 2005). Our sampling indicates that the Eastern clade haplotype has not expanded past areas around the upper ends of Lake Mead.
Patchy Populations and Nonequilibrium Dynamics

The arid environmental conditions between wetland patches within the Mojave Desert generally appear to be quite inhospitable to amphibians. Nevertheless, the data presented herein support a scenario in which *B. punctatus* commonly disperses between neighboring wetland habitats within a mountain range, although we can not firmly reject the hypothesis that migration occurred in the recent past but is not occurring currently. This toad possesses physical and behavioral characteristics that allow it to forage and burrow for substantial periods of time in xeric habitats (see McClanahan et al. 1994). Perhaps dispersal occurs over multiple seasons with dispersing individuals retreating into deep rock crevices or beneath boulders within drainage bottoms during unfavorable seasonal conditions where they maintain water balance within moist soils or sand (Shoemaker 1988). Dispersal across the desert landscape may also be facilitated by extended high precipitation events (Bradford et al. 2003) that create more mesic conditions at intervals of many years to decades within the Mojave Desert (i.e., El Niño/Southern Oscillation).

Our interpretation of migration among sites was also limited because of the maternal inheritance of the mtDNA marker and the potential for sex-biased dispersal. More importantly, the mtDNA sequences only provided a single locus view of genetic structure. Mitochondrial DNA is hypothesized to reveal stronger genetic structure among populations when compared to the nuclear genome because the mtDNA genome has an inherently lower effective population size (Crochet 2000). Differences in the evolutionary dynamics of the two genomes may further inflate mtDNA estimates (e.g., Crawford 2003). These concerns, however, do not affect our interpretations of the
generally low levels of haplotypic structure observed among breeding sites within mountain ranges.

Bradford et al. (2003) posed a scenario that *B. punctatus* expanded into the northeastern Mojave Desert at the end of the Pleistocene when the climate began to dry and warm. As subsequent climatic conditions within the region became more arid, populations became increasingly isolated and clustered among groups of springs within mountain ranges. Our analysis appears to be generally consistent with this scenario. We interpret the overall strong genetic structure associated with mountain ranges as indicating that most populations within ranges currently exchange few, if any, migrants with populations in other mountain ranges. Thus, at scales larger than a single mountain range, nonequilibrium dynamics may predominately describe the current population structure of *B. punctatus* within the Mojave Desert, but within ranges, *B. punctatus* primarily exists within patchy populations.
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Fig. 3.1. Distribution of *Bufo punctatus* in relationship to geographic features within the eastern Mojave Desert around Las Vegas Valley, Nevada. Symbols indicate the results of visual encounter surveys conducted by Bradford et al. (2003) for the presence/absence of this species at springs and rain catchments. Lines emanating from sites indicate drainage channels (mainly dry washes), and enclosed hatched areas are dry lakes.
(playas). Enclosed by thick black lines are seven geographic ranges defined \textit{a priori} on clusters of sites predominately within mountain range groups. Sites from which \( \geq 10 \) individual toads were sampled for genetic analyses are numbered (in relationship to range).
Fig. 3.2. Haplotype network for mtDNA control region sequences of Western clade *Bufo punctatus* constructed under a criterion of statistical parsimony (95% probability criterion)
for connections = 10 steps). Haplotypes are identified by letter designations and surrounded by circles (circles increase in size with haplotype frequency). Numbers following haplotype designations indicate sample sizes greater than one. Within the network, each line between haplotypes represents a mutational change. Small cycles indicate unsampled haplotypes inferred from the data. The five major lineages, discussed within the text, are designated by shading and by the initial letter of the haplotypes contained within (i.e., A – E).
Fig. 3.3. Distribution of *Bufo punctatus* major mtDNA control region clades (pie charts) and haplotypes (identified by letter designations) in relation to seven geographic ranges defined *a priori* on clusters of sites. Numbers following haplotype designations indicate sample sizes for those haplotypes within each range. Asterisks indicate those haplotypes not found in other mountain ranges (i.e., private haplotypes). Arrows indicate pairs of mountain ranges in which a probable stepping-stone connection can be assumed. Pairwise
Φ-statistics between these ranges are indicated next to the arrows (these values were little different from pairwise $F_{ST}$ values derived when samples within each mountain range were pooled, respectively, and treated as populations for comparisons).
Table 3.1. Net average sequence divergences (p-distances) between major control region clades and within-group average divergences (along diagonal).

<table>
<thead>
<tr>
<th></th>
<th>Clade A</th>
<th>Clade B</th>
<th>Clade C</th>
<th>Clade D</th>
<th>Clade E</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.0044</td>
<td>0.0044</td>
<td>0.0134</td>
<td>0.0164</td>
<td>0.0181</td>
</tr>
<tr>
<td>B</td>
<td>0.0045</td>
<td>0.0127</td>
<td>0.0152</td>
<td>0.0163</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>0.0046</td>
<td>0.0112</td>
<td>0.0133</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td>0.0017</td>
<td>0.0085</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0043</td>
</tr>
</tbody>
</table>
Table 3.2. Comparison of control region haplotypes and previously identified cytochrome b haplotypes and associated one-step nested clades identified in Jaeger et al. (2005). Cytochrome b sequence data from Jaeger et al. (2005) or generated for this study (data not shown).

<table>
<thead>
<tr>
<th>Control Region Haplotype (No. compared)</th>
<th>Associated Cytochrome b Haplotype</th>
<th>Associated Cytochrome b Nested Clade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aa (1)</td>
<td>W01</td>
<td>Western clade 1-1</td>
</tr>
<tr>
<td>Ad (5)</td>
<td>W01</td>
<td>Western clade 1-1</td>
</tr>
<tr>
<td>Ae (1)</td>
<td>W01</td>
<td>Western clade 1-1</td>
</tr>
<tr>
<td>Af (1)</td>
<td>W01</td>
<td>Western clade 1-1</td>
</tr>
<tr>
<td>Ba (3)</td>
<td>W03, W04</td>
<td>Western clade 1-4</td>
</tr>
<tr>
<td>Bd (1)</td>
<td>W03</td>
<td>Western clade 1-4</td>
</tr>
<tr>
<td>Be (2)</td>
<td>W03</td>
<td>Western clade 1-4</td>
</tr>
<tr>
<td>Bh (1)</td>
<td>W03</td>
<td>Western clade 1-4</td>
</tr>
<tr>
<td>Ca (1)</td>
<td>W06</td>
<td>Western clade 1-8</td>
</tr>
<tr>
<td>Ch (2)</td>
<td>W02</td>
<td>Western clade 1-7</td>
</tr>
<tr>
<td>Da (2)</td>
<td>Unidentified (related to W07)</td>
<td>(W07 is within clade 1-10)</td>
</tr>
<tr>
<td>Ea (2)</td>
<td>W05</td>
<td>Western clade 1-10</td>
</tr>
<tr>
<td>Eb (1)</td>
<td>W05</td>
<td>Western clade 1-10</td>
</tr>
<tr>
<td>Eastern (3)</td>
<td>E01</td>
<td>Eastern clade 1-1</td>
</tr>
</tbody>
</table>
Table 3.3. Diversity statistics for *Bufo punctatus* mtDNA control region sequence data by geographic range (identified in Fig. 3.1). Fu’s tests are one-tailed with positive values resulting in highly non-significant critical values. Among site Φ values are presented from single-mountain range AMOVAs. Significant values are indicated by bold type.

<table>
<thead>
<tr>
<th>Region</th>
<th>No. Samples (Haplotypes)</th>
<th>Haplotype Diversity ± SD</th>
<th>Nucleotide Diversity ± SD</th>
<th>Tajima’s D (P-value)</th>
<th>Fu’s Fs (P-value)</th>
<th>Among Site Φ (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spring Mountains</td>
<td>241 (9)</td>
<td>0.5556 ± 0.0362</td>
<td>0.0074 ± 0.0041</td>
<td>0.828 (-0.20)</td>
<td>6.206 (0.94)</td>
<td><strong>0.288 (0.00)</strong></td>
</tr>
<tr>
<td>Kingston Range</td>
<td>56 (1)</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clark Mountain</td>
<td>32 (3)</td>
<td>0.1230 ± 0.0777</td>
<td>0.0003 ± 0.0005</td>
<td><strong>-1.730 (0.03)</strong></td>
<td><strong>-1.708 (0.02)</strong></td>
<td>0.000 (1.00)</td>
</tr>
<tr>
<td>McCullough Range</td>
<td>105 (9)</td>
<td>0.7542 ± 0.0252</td>
<td>0.0093 ± 0.0050</td>
<td>1.201 (-0.12)</td>
<td>5.661 (0.94)</td>
<td>0.052 (0.05)</td>
</tr>
<tr>
<td>Eldorado Range</td>
<td>129 (12)</td>
<td>0.8011 ± 0.0218</td>
<td>0.0095 ± 0.0051</td>
<td>0.897 (-0.19)</td>
<td>3.770 (0.88)</td>
<td>0.005 (0.33)</td>
</tr>
<tr>
<td>Black Canyon</td>
<td>139 (10)</td>
<td>0.3882 ± 0.0515</td>
<td>0.0059 ± 0.0034</td>
<td>-0.384 (0.37)</td>
<td>2.197 (0.81)</td>
<td><strong>0.125 (0.00)</strong></td>
</tr>
<tr>
<td>Overton Arm</td>
<td>130 (11)</td>
<td>0.8122 ± 0.0190</td>
<td>0.0095 ± 0.0051</td>
<td><strong>2.155 (-0.02)</strong></td>
<td>4.685 (0.91)</td>
<td><strong>0.242 (0.00)</strong></td>
</tr>
</tbody>
</table>

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Table 3.4. Pairwise $F_{ST}$ values among sites within the Black Canyon area (upper diagonals) and associated $P$-values from permutations tests (lower diagonals). Significant values are indicated by bold type ($P < 0.05$ after sequential Bonferroni correction). Site numbers reference Figure 1 and Appendix.

<table>
<thead>
<tr>
<th>Black Canyon Sites</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>0.188</td>
<td>0.250</td>
<td>0.014</td>
<td>-0.014</td>
<td>0.062</td>
</tr>
<tr>
<td>2</td>
<td>0.035</td>
<td>-</td>
<td>0.000</td>
<td>0.260</td>
<td>0.184</td>
<td>0.017</td>
</tr>
<tr>
<td>3</td>
<td>0.010</td>
<td>0.999</td>
<td>-</td>
<td><strong>0.328</strong></td>
<td><strong>0.245</strong></td>
<td>0.052</td>
</tr>
<tr>
<td>4</td>
<td>0.210</td>
<td>0.006</td>
<td><strong>0.000</strong></td>
<td>-</td>
<td>-0.014</td>
<td>0.150</td>
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<td>5</td>
<td>0.435</td>
<td>0.016</td>
<td><strong>0.002</strong></td>
<td>0.528</td>
<td>-</td>
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<td>0.378</td>
<td>0.237</td>
<td>0.009</td>
<td>0.054</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3.5. Pairwise $F_{ST}$ values among sites within the Spring Range (upper diagonals) and associated $P$-values from permutations tests (lower diagonals). Significant values are indicated by bold type ($P < 0.05$ after sequential Bonferroni correction). Site numbers reference Figure 1 and Appendix.

<table>
<thead>
<tr>
<th>Spring Range Sites</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>0.189</td>
<td>0.877&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.185</td>
<td>0.011</td>
<td>0.024</td>
<td>0.105</td>
<td>0.010</td>
<td>0.137</td>
<td>0.229</td>
<td>0.196</td>
<td>0.114</td>
</tr>
<tr>
<td>2</td>
<td>0.010</td>
<td>–</td>
<td>0.929</td>
<td>0.359&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.060</td>
<td>0.053</td>
<td>0.159</td>
<td>0.133</td>
<td>0.232</td>
<td>0.350&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.287&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.053</td>
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<tr>
<td>3</td>
<td>0.000</td>
<td>0.000</td>
<td>–</td>
<td>0.555&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.812&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.818&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.660&lt;sup&gt;8&lt;/sup&gt;</td>
<td>0.875&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.588&lt;sup&gt;10&lt;/sup&gt;</td>
<td>0.455&lt;sup&gt;11&lt;/sup&gt;</td>
<td>0.518&lt;sup&gt;12&lt;/sup&gt;</td>
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<td>4</td>
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<td>0.000</td>
<td>0.001</td>
<td>–</td>
<td>0.121&lt;sup&gt;14&lt;/sup&gt;</td>
<td>0.128&lt;sup&gt;15&lt;/sup&gt;</td>
<td>–</td>
<td>0.010&lt;sup&gt;16&lt;/sup&gt;</td>
<td>0.241&lt;sup&gt;17&lt;/sup&gt;</td>
<td>–</td>
<td>0.016&lt;sup&gt;18&lt;/sup&gt;</td>
<td>0.081&lt;sup&gt;19&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>0.256&lt;sup&gt;20&lt;/sup&gt;</td>
<td>0.013</td>
<td>0.000</td>
<td>0.027</td>
<td>–</td>
<td>0.009&lt;sup&gt;21&lt;/sup&gt;</td>
<td>0.019&lt;sup&gt;22&lt;/sup&gt;</td>
<td>0.024&lt;sup&gt;23&lt;/sup&gt;</td>
<td>0.074&lt;sup&gt;24&lt;/sup&gt;</td>
<td>0.190&lt;sup&gt;25&lt;/sup&gt;</td>
<td>0.124&lt;sup&gt;26&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>0.244</td>
<td>0.027</td>
<td>0.000</td>
<td>0.028</td>
<td>0.999&lt;sup&gt;28&lt;/sup&gt;</td>
<td>–</td>
<td>0.016&lt;sup&gt;29&lt;/sup&gt;</td>
<td>0.041&lt;sup&gt;30&lt;/sup&gt;</td>
<td>0.081&lt;sup&gt;31&lt;/sup&gt;</td>
<td>0.206&lt;sup&gt;32&lt;/sup&gt;</td>
<td>0.131&lt;sup&gt;33&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>0.070</td>
<td>0.005</td>
<td>0.000</td>
<td>0.398</td>
<td>0.202&lt;sup&gt;35&lt;/sup&gt;</td>
<td>0.329&lt;sup&gt;36&lt;/sup&gt;</td>
<td>–</td>
<td>0.122&lt;sup&gt;37&lt;/sup&gt;</td>
<td>–</td>
<td>0.001&lt;sup&gt;38&lt;/sup&gt;</td>
<td>0.089&lt;sup&gt;39&lt;/sup&gt;</td>
<td>0.015&lt;sup&gt;40&lt;/sup&gt;</td>
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<tr>
<td>8</td>
<td>0.313</td>
<td>0.000</td>
<td>0.000</td>
<td>0.002</td>
<td>0.186&lt;sup&gt;42&lt;/sup&gt;</td>
<td>0.060&lt;sup&gt;43&lt;/sup&gt;</td>
<td>0.029&lt;sup&gt;44&lt;/sup&gt;</td>
<td>–</td>
<td>0.165&lt;sup&gt;45&lt;/sup&gt;</td>
<td>0.261&lt;sup&gt;46&lt;/sup&gt;</td>
<td>0.228&lt;sup&gt;47&lt;/sup&gt;</td>
<td>0.058&lt;sup&gt;48&lt;/sup&gt;</td>
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<td>9</td>
<td>0.026</td>
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<td>0.000</td>
<td>0.499&lt;sup&gt;49&lt;/sup&gt;</td>
<td>0.063&lt;sup&gt;50&lt;/sup&gt;</td>
<td>0.057&lt;sup&gt;51&lt;/sup&gt;</td>
<td>0.317&lt;sup&gt;52&lt;/sup&gt;</td>
<td>0.004&lt;sup&gt;53&lt;/sup&gt;</td>
<td>–</td>
<td>0.023&lt;sup&gt;54&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>0.003</td>
<td>0.000</td>
<td>0.000</td>
<td>0.425&lt;sup&gt;56&lt;/sup&gt;</td>
<td>0.002&lt;sup&gt;57&lt;/sup&gt;</td>
<td>0.002&lt;sup&gt;58&lt;/sup&gt;</td>
<td>0.044&lt;sup&gt;59&lt;/sup&gt;</td>
<td>0.000&lt;sup&gt;60&lt;/sup&gt;</td>
<td>0.223&lt;sup&gt;61&lt;/sup&gt;</td>
<td>–</td>
<td>0.002&lt;sup&gt;62&lt;/sup&gt;</td>
<td>0.254&lt;sup&gt;63&lt;/sup&gt;</td>
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<td>11</td>
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<td>0.000</td>
<td>0.533&lt;sup&gt;64&lt;/sup&gt;</td>
<td>0.029&lt;sup&gt;65&lt;/sup&gt;</td>
<td>0.025&lt;sup&gt;66&lt;/sup&gt;</td>
<td>0.228&lt;sup&gt;67&lt;/sup&gt;</td>
<td>0.001&lt;sup&gt;68&lt;/sup&gt;</td>
<td>0.692&lt;sup&gt;69&lt;/sup&gt;</td>
<td>0.343&lt;sup&gt;70&lt;/sup&gt;</td>
<td>–</td>
<td>0.193&lt;sup&gt;71&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>0.234&lt;sup&gt;72&lt;/sup&gt;</td>
<td>0.299&lt;sup&gt;73&lt;/sup&gt;</td>
<td>0.000</td>
<td>0.040&lt;sup&gt;74&lt;/sup&gt;</td>
<td>0.387&lt;sup&gt;75&lt;/sup&gt;</td>
<td>0.688&lt;sup&gt;76&lt;/sup&gt;</td>
<td>0.293&lt;sup&gt;77&lt;/sup&gt;</td>
<td>0.136&lt;sup&gt;78&lt;/sup&gt;</td>
<td>0.035&lt;sup&gt;79&lt;/sup&gt;</td>
<td>0.005&lt;sup&gt;80&lt;/sup&gt;</td>
<td>0.030&lt;sup&gt;81&lt;/sup&gt;</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 3.6. Pairwise $F_{ST}$ values among sites within the Overton Arm area (upper diagonals) and associated $P$-values from permutations tests (lower diagonals). Significant values are indicated by bold type ($P < 0.05$ after sequential Bonferroni correction). Site numbers reference Figure 1 and Appendix.

<table>
<thead>
<tr>
<th>Overton Arm Sites</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>0.227</td>
<td>0.015</td>
<td>0.432</td>
<td>0.103</td>
<td>0.368</td>
<td>0.259</td>
<td>0.073</td>
</tr>
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<td>0.033</td>
<td>0.675</td>
<td>0.326</td>
<td>0.622</td>
<td>0.466</td>
<td>0.264</td>
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<tr>
<td>3</td>
<td>0.268</td>
<td>0.186</td>
<td>–</td>
<td>0.456</td>
<td>0.080</td>
<td>0.397</td>
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<td>0.088</td>
</tr>
<tr>
<td>4</td>
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<td>0.000</td>
<td>0.008</td>
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<td>0.115</td>
<td>-0.062</td>
<td>-0.008</td>
<td>0.317</td>
</tr>
<tr>
<td>5</td>
<td>0.091</td>
<td>0.001</td>
<td>0.120</td>
<td>0.181</td>
<td>–</td>
<td>0.054</td>
<td>-0.007</td>
<td>0.016</td>
</tr>
<tr>
<td>6</td>
<td>0.002</td>
<td>0.000</td>
<td>0.003</td>
<td>0.670</td>
<td>0.190</td>
<td>–</td>
<td>-0.021</td>
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<td>7</td>
<td>0.002</td>
<td>0.000</td>
<td>0.006</td>
<td>0.376</td>
<td>0.355</td>
<td>0.444</td>
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<td>0.164</td>
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<td>0.089</td>
<td>0.007</td>
<td>0.247</td>
<td>0.020</td>
<td>0.017</td>
<td>–</td>
</tr>
</tbody>
</table>

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Appendix. Descriptions of sample locations, sample sizes, and haplotype numbers by range and site number referenced in Figure 1. Latitude and longitude (in parentheses following site numbers) are presented in decimal degrees projected to datum NAD 1983 (longitudes are negative). Haplotypes and counts follow sample sizes (in parentheses).

Black Canyon: Site 1 (36.0119, 114.7462), n = 25 (Ba 18, Ch 7); Site 2 (36.0023, 114.7436), n = 14 (Ba 14); Site 3 (35.9853, 114.7489), n = 25 (Ba 25); Site 4 (35.9408, 114.7351), n = 25 (Ba 13, Be 1, Ch 1, Ci 7, Cg 1, Ed 2); Site 5 (35.8505, 114.7267), n = 25 (Ba 16, Ca 3, Cc 1, Ci 3, Ec 2); Site 6 (35.9624, 114.7266), n = 25 (Ba 22, Cd 1, Ci 1, Ed 1). Clark Mountain: Site 1 (35.5559, 115.5549), n = 16 (Aa 1, Ad 15); Site 2 (35.5246, 115.5374), n = 16 (Ad 15, Ag 1). Eldorado Mountains: Site 1 (35.7291, 114.8212), n = 25 (Ba 7, Bj 1, Ca 4, Cb 2, Cc 5, Ce 1, Da 4, Ed 1); Site 2 (35.6997, 114.8651), n = 15 (Ba 7, Ca 1, Cb 1, Cc 6); Site 3 (35.7066, 114.8118), n = 25 (Ba 11, Bj 2, Cc 10, Ce 1, Da 1); Site 4 (35.6629, 114.8060), n = 25 (Ba 6, Bd 1, Bj 1, Ca 5, Cb 1, Cc 10, Ch 1); Site 5 (35.6615, 114.7726), n = 25 (Ba 3, Bd 3, Bj 2, Cc 6, Ce 6, Ch 2, Ed 3); Site 6 (35.6227, 114.8443), n = 14 (Ba 5, Bd 1, Bm 1, Cc 3, Cc 2, Db 1, Ed 1).

Kingston Range: Site 1 (35.7854, 115.9330), n = 16 (Ad 16); Site 2 (35.7969, 115.9627), n = 16 (Ad 16); Site 3 (35.7743, 115.8896), n = 14 (Ad 14). McCullough Range (includes Highland Range): Site 1 (35.6350, 115.1970), n = 25 (Ad 8, Ae 5, Ba 1, Bd 2, Ca 9); Site 2 (35.6060, 115.2017) n = 10 (Aa 1, Ad 1, Ae 2, Ba 1, Ca 5); Site 3 (35.6501, 115.1301), n = 24 (Ad 2, Ae 2, Ba 10, Ca 10); Site 4 (35.5981, 115.0600), n = 20 (Aa 1, Af 1, Ba 9, Ca 9); Site 5 (35.5695, 115.0162), n = 15 (Af 2, Ba 8, Bd 2, Ca 2, Ch 1); Site 6 (35.4796, 115.1742), n = 11 (Ad 3, Ae 1, Ca 5, Ea 2). Overton Arm: Site 1 (36.6904, 114.7077), n = 20 (Ba 1, Bh 6, B1 9, Ch 4); Site 2 (36.4887, 114.4675), n = 20 (Bd 2, Be 15, B1 2, Cj 1); Site 3 (36.4399, 114.5130), n = 12 (Ba 1, Bd 1, Be 5, Bl 3, Ch 2); Site 4 (36.4028, 114.4026), n = 10 (Be 1, Bl 1, Ch 8); Site 5 (36.3766, 114.4494), n = 11 (Ba 2, Be 2, Bh 1, Bl 1, Ch 5); Site 6 (36.3538, 114.4172), n = 25 (Ba 21, Bb 1, Bg 1, Ea 2); Site 7 (36.2871, 114.5147), n = 24 (Bd 4, Be 1, Bf 1, Bl 3, Ch 15); Site 8 (36.4608, 114.2594), n = 22 (Ba 5, Bh 9, Bi 1, Ca 1, Cf 1, Cj 5). Spring Mountain Range: Site 1 (36.2989, 115.8726), n = 14 (Aa 11, Bg 3); Site 2 (36.1671, 115.7260), n = 25 (Aa 21, Ab 4); Site 3 (36.1102, 115.7364), n = 14 (Aa 1, Eb 13); Site 4 (36.0052, 115.4923), n = 11 (Aa 6, Bk 2, Eb 3); Site 5 (36.0538, 115.4172), n = 25 (Ba 21, Bb 1, Bg 1, Ea 2); Site 6 (36.1039, 115.4826), n = 25 (Aa 22, Bg 1, Ea 1, Eb 1); Site 7 (36.1233, 115.4933), n = 25 (Aa 19, Ac 1, Ea 1, Eb 4); Site 8 (36.1580, 115.4970), n = 22 (Aa 14, Ac 4, Bb 3, Bg 1); Site 9 (36.1759, 115.4804), n = 21 (Aa 11, Ac 2, Bg 1, Ca 3, Ea 4); Site 10 (36.1604, 115.4318), n = 25 (Aa 7, Bb 7, Bg 1, Ca 2, Ea 7, Eb 1); Site 11 (36.1841, 115.4302), n = 24 (Aa 14, Bb 1, Ca 2, Ea 7); Site 12 (35.8911, 115.3718), n = 10 (Aa 10).
CHAPTER IV

EVIDENCE FOR PHYLOGENETICALLY DISTINCT LEOPARD FROGS

(*RANA ONCA*) FROM THE BORDER REGION OF NEVADA,
UTAH, AND ARIZONA

Abstract

Remnant populations of leopard frogs within the Virgin River drainage and adjacent portions of the Colorado River (Black Canyon) in northwestern Arizona and southern Nevada either represent the reportedly extinct taxon *Rana onca* or northern, disjunct *R. yavapaiensis*. To determine the evolutionary distinctiveness of these leopard frogs, we evaluated mitochondrial DNA (mtDNA) restriction site variation (RFLP), mtDNA control region sequences, randomly amplified polymorphic DNA (RAPD) markers, and morphological characters. Individuals from the Virgin River drainage and Black Canyon represented a single RFLP haplotype and were identical for nucleotides along a portion of control region sequence. Evaluations of RAPD data demonstrated high levels of similarity among individuals and populations from this region. Leopard frogs from the Virgin River drainage and Black Canyon differed from *R. yavapaiensis* from west-central Arizona and northern Mexico in maximum parsimony and distance analyses of RFLP and control region sequence data, and in maximum likelihood analysis of the
sequence data. Multidimensional scaling of RAPD data provided a similar and congruent indication of this separation. Analysis of principal component scores demonstrated significant morphological differentiation between leopard frog specimens from the Virgin River drainage and \textit{R. yavapaiensis}. Parallel patterns of divergence observed in the mtDNA, RAPD, and morphological analyses indicate that leopard frogs from the Virgin River drainage and adjacent portions of the Colorado River are phylogenetically distinct. These leopard frogs should be recognized as a lineage separate from southern populations of \textit{R. yavapaiensis} and classified as the species \textit{R. onca}.

Introduction

Controversy over the taxonomic validity and evolutionary distinctiveness of leopard frog species (\textit{Rana pipiens} complex) in southern Nevada, southwestern Utah, and northwestern Arizona has confounded efforts to understand the conservation implications of the loss and decline of populations within this region. \textit{Rana onca} Cope, the relict leopard frog, was described in 1875 from a single adult female likely collected along the Virgin River in Washington County, Utah (Cope 1875 \textit{in} Tanner 1929). Several years later, \textit{R. fisheri} Stejneger, the Vegas Valley leopard frog, was described from a series of specimens collected from springs within the Las Vegas Valley, Nevada (Stejneger 1893). The taxonomic relationship between these two nominal species has been a source of contention. Many authors considered \textit{R. fisheri} and \textit{R. onca} as synonyms (see Jennings 1988 for citations), but actual comparisons between the two taxa were few and suffered from a perceived paucity of \textit{R. onca} specimens (Slevin 1928; Pace, 1974). Other authors,
however, clearly thought the synonomy was not warranted (Linsdale 1940; Wright and Wright 1949; Stebbins 1951).

Populations of *R. fisheri* within the Las Vegas Valley are thought to have gone extinct in the late 1940's due to habitat alterations (Stebbins 1951). *Rana onca* populations along the Virgin River drainage were thought to have gone extinct sometime after 1950 (Jennings 1988; J. E. Platz, Status report for *Rana onca* Cope, U.S. Fish and Wildlife Service 1984, unpublished). Leopard frogs persist at sites along the Virgin River from Littlefield, Arizona downstream to areas in the Black Canyon along the Colorado River (Fig. 4.1), and furthering the taxonomic confusion, some of these populations (e.g., Littlefield, Arizona) were more recently considered disjunct populations of *Rana yavapaiensis* Platz and Frost, the lowland leopard frog (Platz and Frost 1984). *Rana yavapaiensis* exhibits a relatively continuous distribution extending from Sonora, Mexico into southern and central Arizona and southwestern New Mexico, with additional populations (now thought to be extinct) in a region centered around the Imperial Valley of southern California (Fig. 4.1; Platz and Frost 1984; Platz 1988; Jennings and Hayes 1994). *Rana pipiens* Schreber, the northern leopard frog (Schreber 1782 in Pace 1974), also occurs within the upper reaches of the Virgin River, but this species is extralimital to this study.

Leopard frogs from the extant populations along the Virgin River drainage are morphologically variable, ranging from those that match the description and appearance of the type specimen of *R. onca* to those that more closely resemble *R. yavapaiensis* from southern localities (see morphological analyses below). One hypothesis to explain the presence of extant, morphologically variable leopard frogs in the Virgin River drainage is
that two leopard frog taxa, and perhaps their hybrids, currently occur within the lower portions of the drainage. Alternatively, leopard frogs within this region may represent a single, morphologically variable taxon. In this paper, we use molecular and morphological evidence to evaluate these hypotheses and therefore determine the evolutionary distinctiveness of Virgin River populations with respect to *R. yavapaiensis* populations to the south. The question of the identity of extinct leopard frogs from the Las Vegas Valley (i.e., *R. fisheri*) will be dealt with elsewhere. We conclude with a consideration of the taxonomy of leopard frogs from the Virgin River drainage and adjacent portions of the Colorado River drainage in light of our genetic and morphological analyses.

**Materials and Methods**

**Samples**

Extensive surveys revealed seven sites in three general areas of the Virgin River drainage and adjacent portions of the Colorado River that currently contain leopard frogs: Littlefield, Arizona; Overton Arm of Lake Mead; and Black Canyon along the Colorado River (Fig. 4.1; henceforth referred to as “Virgin River/Black Canyon”). All seven sites are perennial spring-fed habitats. Collections for genetic analyses were made at all sites during the 1990’s, but no frogs have been found at Corral Spring (Fig. 4.1) since 1995. Leopard frogs representing *R. yavapaiensis* from the more southern contiguous range were collected from three locations in west-central Arizona and one site in northern Mexico (Fig. 4.1; henceforth, all samples from Trout Creek south into Mexico will be referred to as “*R. yavapaiensis*”). Other southwestern ranid species (*R. berlandieri, R.*
blairi, R. chiricahuensis, and R. pipiens) were included in the mtDNA analyses (see Materials Examined for collection localities). Muscle, heart, or liver tissue of sacrificed adult animals or toe tips from animals captured and then released were used to isolate genomic DNA in phenol-chloroform-isoamyl alcohol extractions.

Samples for morphological analyses were museum specimens of adult leopard frogs housed in eight regional and national collections (see Materials Examined). Examined specimens included: 53 leopard frogs from the Virgin River drainage west of Hurricane, Utah downstream to the Overton Arm of Lake Mead in Nevada (including the type specimen of R. onca); 5 leopard frogs from the Imperial Valley of California; and 25 R. yavapaiensis. To better interpret morphological variation in visual analyses, specimens from the Virgin River drainage were further assigned geographical designations: Overton Arm of Lake Mead or Virgin River drainage upstream of Lake Mead. Most of the samples from sites along the Overton Arm of Lake Mead were collections from extant populations. We also, a priori, assigned a designation to those specimens from upstream locations that upon visual inspection matched the description and appearance of the type specimen of R. onca.

Mitochondrial DNA Restriction Site Variation

A total of 50 southwestern leopard frog specimens representing six nominate species were assayed for mtDNA restriction-site variation (restriction fragment-length polymorphisms; RFLP). Of these samples, 19 animals were from six of the Virgin River/Black Canyon sites and 11 animals were from three sites within the southern range of R. yavapaiensis. Oligonucleotide primers (Riddle et al. 1993) located in the met-tRNA (L3880) and cytochrome oxidase subunit 1 (COI) genes (H6033) were used in polymerase
chain reaction (PCR) to amplify a fragment approximately 2150 basepairs (bp) in size that included the NADH subunit 2 (ND2) gene (about 1035 bp), five intervening tRNAs, and about 705 bp of the COI gene.

Eleven tetra- or heptanucleotide restriction enzymes were used in the final analysis (Bsp1286I, BstUI, DpnII, HaeIII, Hhal, HinfI, MspI, Rsal, Sau961A and TaqI). Each restriction digestion was conducted using around 7µL of PCR product according to manufacturers’ protocols (New England Biolabs, Inc.). Digests were electrophoresed through a 2.0% agarose gel and visualized using ethidium bromide staining. Digital photographs of each gel were made for analyses, and restriction fragment sizes were estimated by visual comparisons against molecular-weight markers run on each gel. Restriction-site gains and losses were inferred for each enzyme through direct examination of fragment patterns under the assumption that comigrating fragments from different specimens represented identical stretches of mtDNA, and that fragment patterns that differed minimally by presence or absence of two fragments could be attributed to at least one restriction-site gain or loss.

Phylogenetic analyses were performed using PAUP* (Phylogenetic Analysis Using Parsimony and Other Methods, vers. 4.0b; Swofford 1998). *Rana chiricahuensis* was selected as an outgroup based on prior evidence of a distant relationship between this species and all others examined herein (Hillis 1988). Neighbor-joining (NJ) trees (Saitou and Nei 1987) were constructed using a matrix of sequence divergence estimates among haplotypes (Nei and Li 1979). Two separate maximum parsimony (MP) analyses were conducted under different character weighting assumptions: site gains and losses weighted equally (Wagner parsimony; Farris 1970), and site gains constrained to occur
only once while multiple losses were allowed (Dollo parsimony; DeBry and Slade 1985). Nonparametric bootstrap values were generated as a depiction of the robustness of clades on NJ and MP trees (Felsenstein 1985; Hillis and Bull 1993). Wilcoxon signed-rank tests (Templeton 1983) were used to evaluate null hypotheses of no difference ($P < 0.05$) between the best MP tree and less-parsimonious user-input alternative trees.

Control Region Sequence Variation

Nine leopard frogs from the seven Virgin River/Black Canyon sites, six leopard frogs representing *R. yavapaiensis* from four sites in west-central Arizona and northern Mexico, and representative samples of *R. berlandieri*, *R. blairi*, *R. chiricahuensis*, and *R. pipiens* were sequenced for a portion of the mtDNA control region. Primers CytbA-L and ControlP-H from Goebel et al. (1999) were used to PCR-amplify and sequence a segment of the mtDNA that was generally either 1137 bp or 1224 bp long in *R. yavapaiensis*, *R. blairi*, *R. berlandieri* and Virgin River/Black Canyon samples, depending on the number of repeat elements and insertion/deletions. Sequences were considerably longer in *R. chiricahuensis* and *R. pipiens* (see below). Two additional sequencing primers were designed within the control region: Hrana-1232 (TCT GCG TGA TCT AAT GCA AG) was used to sequence the light-strand spanning the gap between sequences from CytbA-L and ControlP-H, and HranaA-L (GTG TAG ATA TTR AGA TGG GTA TC) was used to sequence a strand mostly complementary to that from CytbA-L.

Sequences were determined using an ABI Prism 310 automated sequencer and Big Dye Terminator Cycle Sequencing chemistry (PE Applied Biosystems, Inc.). Chromatograms were evaluated and corrected by eye. In addition to the control region sequence, raw sequences contained about 103-106 bp of cytochrome b (cyt b). This
region was aligned with published *R. catesbeiana* sequence (Yoneyama 1987). Virgin River/Black Canyon and *R. yavapaiensis* sequences contained one additional amino acid at the three-prime end prior to the terminator. Relatively low rates of nucleotide substitution in the cyt b gene appear to be common in amphibians (Graybeal 1993; Caccone et al. 1997), but the control region contains many sites that are non-coding and evolve quickly (see Taberlet 1996). Because of the difference in evolution between these mtDNA regions, cyt b sequences and terminators were excluded from analyses.

In all samples the beginning of the control region consisted of a recurrent element in various forms. In its shortest form (*R. berlandieri, R. blairi, and most R. yavapaiensis* and Virgin River/Black Canyon samples), the recurrent element consisted generally of 87 bp, with some sequence variation, repeated twice with a portion of the recurrent element repeated again downstream. Some *R. yavapaiensis* and Virgin River/Black Canyon sequences contained an additional version of the repeat element, and the beginning of the control region in both the *R. chiricahuensis* and *R. pipiens* consisted of longer segments that appeared to contain portions of the recurrent element. Final alignments of control region sequences were constructed using ClustalW as implemented in BioEdit (vers. 4.8.7: Hall 1999). To allow meaningful comparisons of sequence data, additional repeat elements within some of the *R. yavapaiensis* and Virgin River/Black Canyon samples, along with 399 bp and 236 bp at the beginning of the *R. chiricahuensis* and *R. pipiens* sequences, respectively, were discarded prior to analyses. In this form, control region sequences included in the analysis ranged from 948 (*R. chiricahuensis*) to 962 bp with most sequences being 959 bp in size.
Maximum likelihood (ML), MP and NJ analyses were used to evaluate patterns of control region sequence divergence. All analyses were conducted using PAUP* with gaps ignored and *R. chiricahuensis* designated as an outgroup. A NJ tree (Saitou and Nei 1987) was first constructed using the Tamura-Nei (1993) model of nucleotide substitution. Maximum parsimony trees were generated using the branch and bound algorithm under equal weighting of all character changes. Bootstrap values were calculated to evaluate support for various clades within NJ and MP trees. Sequence data were evaluated using Modeltest (Ver. 3.0; Posada and Crandall 1998) to choose an appropriate model of sequence evolution for use in a heuristic search using the ML criterion. Modeltest uses a hierarchical approach and likelihood ratio tests to evaluate the best fit of data to a series of DNA substitution models.

**RAPD Variation**

Randomly amplified polymorphic DNA (RAPD) data were generated following the general outline of Williams et al. (1990). Because RAPD-PCR is sensitive to variation in reaction conditions, only samples from which DNA was recently extracted from toe tips were used in analyses. We included 102 samples from six Virgin River/Black Canyon sites and 19 *R. yavapaiensis* from two locations.

RAPD-PCR was performed in 25 μL reactions containing nuclease-free water, 3 mM MgCl₂, 10 mM Tris-HCl, 10 mM KCl, 0.2 mM of each dNTP, 1 μM of a single 10 bp oligonucleotide primer, 1.25 unit of Taq DNA polymerase Stoffel Fragment (PE Applied Biosystems, Inc.), and approximately 30 ng of DNA. Thermal cycling consisted of an initial denaturing step of 94 °C for 2.5 min, followed by 44 repetitions of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min. The 72 °C extension was held for 10 additional
minutes during the final cycle prior to being held at 4 °C. Amplification products were visualized on 2% agarose gels run for approximately 4.3 hours at 93 volts and detected with ethidium bromide under UV light. Digital images of the gels were taken and printouts of these images were used for fragment scoring. Size standards (usually 100 bp ladder) were run on each gel and used to estimate fragment sizes.

A preliminary screening of 134 primers was conducted with a subsample of five or six leopard frogs representing geographically distinct sites. Primers were from random 10 bp primer kits (Operon Technologies, Inc) or were identified as useful in a previous RAPD analysis of *R. pipiens* (Kimberling et al. 1996). Further analyses were conducted with primers that produced relatively unambiguous fragments and had at least one polymorphic fragment (marker) between any of the samples.

Markers were identified by primer name and fragment size and each marker was scored as present (1) or absent (0). Over the course of days necessary to collect data using a particular primer, various samples were subject to repeated PCR’s to confirm that markers were consistent (average = 18 samples/primer). Any marker not consistently scored between these successive PCR’s was discarded; this resulted in some primers being discarded from further analysis. Only polymorphic markers showing repeatable, high intensity amplifications were scored. This conservative approach to marker selection resulted in the scoring of 10 primers for 21 markers (Table 4.1).

Similarity between two samples was calculated using the Dice formula (Nei and Li 1979) as implemented by the program NTSYSpc (Numerical Taxonomy and Multivariate Analysis System, vers. 2.0; Rohlf 1998). Index values for this measure
range from 1 (identical) to 0 (no similarity). Similarity between two sample sites was calculated using the formula:

$$\bar{S}_y = 1 + \bar{S}_i - \frac{\bar{S}_i + \bar{S}_j}{2}$$

where $\bar{S}_i$ and $\bar{S}_j$ are the average similarity between individuals within sample sites $i$ and $j$, respectively, and $\bar{S}_y$ is the average similarity between individuals among sample sites $i$ and $j$ (Wright 1965; Liao and Hsiao 1998). Using this formula, the index of inter-site similarity is corrected by intra-site similarities. Multidimensional scaling (MDS) analyses based on the pairwise similarity matrixes were performed as implemented in NTSYSpc. Because problems with multiple local minima is possible in MDS, principle coordinates analysis (PCoA) was first conducted on each data set and used as the initial configuration matrix for the respective MDS analysis. Using the results from a PCoA as an initial configuration matrix assures that the results from MDS will not be worse than that of a PCoA analysis (Rohlf 1998).

**Morphological Analyses**

Eight continuous morphological traits were measured and 11 discontinuous traits were coded on each museum specimen. Coding protocols for discrete characters were developed to create an ordinal series of character states (see Appendix I for coding protocols). Continuous traits were head width, head length, lip height, internarial distance, tympanum diameter, eye diameter, tibiofibula length, and snout-urostyle length. Discontinuous traits were the condition of the dorsolateral folds, number of spots anterior to the eyes, number of spots on the head above the eyes, number of dorsal spots between the dorsolateral folds, number of bars on the dorsal surface of the thigh, condition of the
thigh pattern, condition of the tympanum spot, degree of the mottling on the lower lip, degree of mottling on the chin, condition of the supralabial stripe, and extent of the webbing on the hind foot.

The nineteen morphologic traits were subjected to principal components analysis (PROC FACTOR; SAS Ver. 6, SAS Institute Inc. 1985. Cary, NC) in order to understand better the relationships among variables and to identify major trends in the data. Continuous and discontinuous data were ranked prior to analysis to reduce non-normality (Conover 1980; Conover and Iman 1981) and to allow both types of data to be used in the same analysis. Principal components satisfying a minimum eigenvalue-equal-one criterion were plotted against each other using species/geographical designations as markers to elucidate major groups of leopard frogs (cluster analysis). Initially, cluster plots were performed separately for each sex to eliminate conflicting patterns between the sexes. Principal component scores for individual frogs were subjected to a two-way analysis of variance with interaction using major species/geographical designation (all samples from the Virgin River drainage were assigned a single geographical designation) and sex as class variables (PROC GLM; SAS Ver. 6) followed by pairwise comparisons of least-squared means.

Results

Restriction Site Variation

From assay of restriction site variation, 12 composite mtDNA haplotypes were inferred among 50 individuals representing six nominate species (see Appendix II for haplotypes and restriction site variation data). Presence or absence of distinct restriction
sites could generally be inferred through examination of variable fragment patterns. This approach could not be employed to deduce homologous restriction sites between *R. chiricahuensis* and other species for three restriction enzymes because of increased RFLP pattern complexity at this level of divergence. These characters were therefore coded as ‘missing information’ in *R. chiricahuensis*. A total of 60 restriction sites were thus recorded, 40 being variable excluding *R. chiricahuensis*. All individuals (n = 19) from the Virgin River/Black Canyon sites possessed a single haplotype not found elsewhere (01 Virgin River/Black Canyon). Five haplotypes were found in 11 individuals from the three southern populations of *R. yavapaiensis* (02-06 *R. yavapaiensis*). None of the *R. yavapaiensis* haplotypes were shared with other species of leopard frogs. A total of six additional haplotypes were distributed among *R. blairi*, *R. berlandieri*, *R. chiricahuensis*, and *R. pipiens*. Pairwise estimates of sequence divergence among haplotypes (excluding *R. chiricahuensis*) ranged from low values of 0.2% (between haplotypes 09 and 10) and 0.3% (haplotypes 03,04; 03,05; 05,06) to high values of about 10-11% between *R. pipiens* haplotypes 09 and 10 and any of the *R. yavapaiensis* and Virgin River/Black Canyon haplotypes (Table 4.2).

Parsimony analysis performed under Wagner and Dollo criteria produced MP trees (Fig. 4.2A) that were identical in topology for major clades (Wagner: length = 57, CI = 0.77, RI = 0.88; Dollo: length = 60, CI = 0.73; RI = 0.94). These trees indicated a monophyletic clade uniting haplotypes 02-06 *R. yavapaiensis* with haplotype 01 Virgin River/Black Canyon, a separate *R. berlandieri* + *R. blairi* clade, and a basal *R. pipiens* clade (Fig. 4.2A). Both Wagner and Dollo MP trees indicated a clade consisting of *R. yavapaiensis* haplotypes 02-06 relative to the Virgin River/Black Canyon haplotype 01,
but this relationship received stronger bootstrap support under Dollo parsimony (Bootstrap = 82). Statistical evaluations (one-tailed Wilcoxon signed-rank tests) of the MP tree against user-input alternative trees that joined haplotypes 01 Virgin River/Black Canyon and 02 *R. yavapaiensis* or 06 *R. yavapaiensis* into a clade indicated that the alternative trees were significantly worse under both Wagner (haplotypes 01 with 02: length = 59, $P = 0.03$; haplotypes 01 with 06: length = 59, $P = 0.03$) and Dollo (haplotypes 01 with 02: length = 63, $P = 0.04$; haplotypes 01 with 06: length = 63, $P = 0.04$) parsimony. If the user-input tree further eroded the *R. yavapaiensis* + Virgin River/Black Canyon clade, by joining haplotype 01 with the *R. berlandieri* + *R. blairi* clade, the alternative tree was significantly worse than the original MP tree under both Wagner (length = 67, $P = 0.0008$) and Dollo (length = 72, $P = 0.00025$) parsimony.

A NJ tree (not shown) constructed using the distance matrix (Table 4.2) was consistent with MP trees in indicating a monophyletic clade uniting haplotypes 02-06 *R. yavapaiensis* with haplotype 01 Virgin River/Black Canyon. This clade was established in 100% of the bootstrap replicates. A clade consisting of *R. yavapaiensis* haplotypes 02-06 relative to the haplotype 01 Virgin River/Black Canyon was supported, but only weakly (bootstraps = 68%). The NJ tree differed from MP trees by weakly supporting a clade consisting of *R. berlandieri*, *R. blairi* and *R. pipiens* (bootstraps < 50%).

### Control Region Sequence Variation

Sequence data consisted of eight haplotypes (including the outgroup). Control region sequence nucleotides were identical for all nine individuals from the seven Virgin River/Black Canyon sites (haplotype 01; Table 4.3). Three haplotypes were identified from the six individuals representing *R. yavapaiensis* populations with haplotypes...
differing from each other by Tamura-Nei distances of 0.0031 to 0.0052 (haplotypes 02-04; Table 4.3). The Virgin River/Black Canyon haplotype differed from sequences of *R. yavapaiensis* by 42 to 45 nucleotide changes which resulted in Tamura-Nei distances of 0.0456 to 0.049 (Table 4.3). All other haplotypes were representative of the species included in the analyses.

Evaluation of the sequence data using Modeltest indicated that the HKY85 (Hasegawa et al. 1985) model with gamma distributed rate heterogeneity was an appropriate model of nucleotide substitution. Using this model, a heuristic search (random addition, one replication, tbr branch swapping) recovered an ML tree (-InL score = 3189.17428, \( \alpha = 0.449607 \)) with a topology nearly identical to that produced from RFLP MP analyses (Fig. 4.2B). Neighbor-joining and MP analyses both produced trees with nearly identical topologies to the ML tree. Support was strong (Bootstraps = 100%) in both the NJ and MP analyses for major clades and for a monophyletic relationship between *R. yavapaiensis* haplotypes (02-04) and the Virgin River/Black Canyon haplotype (01).

**RAPD Variation**

Estimates of average within population similarity between pairs of individuals derived from RAPD data were high for Virgin River/Black Canyon sites (sites 1-6; Table 4.4). Among-population similarity within this region was also high (similarity between any pair of Virgin River/Black Canyon sites was \( \geq 0.918 \); Table 4.4). The two *R. yavapaiensis* populations (sites 7-8; Table 4.4) showed somewhat lower average similarity for pairs of samples within each population, but the similarity between these two populations was high (similarity = 0.938). Similarity values decreased markedly in
contrasts between Virgin River/Black Canyon sites and the two *R. yavapaiensis* populations (average similarity between pairs of Virgin River/Black Canyon sites and *R. yavapaiensis* sites ranged from 0.481 to 0.570; Table 4.4).

The MDS analysis based on pairwise similarity between individuals demonstrated the divergence between Virgin River/Black Canyon frogs and *R. yavapaiensis* using two dimensions (Figs. 3A). A measure of the goodness-of-fit between MDS results (distances in the configuration space) and the monotone function of the original distances was good (Stress 2 = 0.068; Rohlf 1998). A complementary pattern was obtained from the MDS analysis based on the pairwise similarity between sample sites (Stress 2 = 0.008; Fig. 3B). Further interpretation of the RAPD data to derive estimates of the relationships among Virgin River/Black Canyon sample sites was not considered useful. Of the 21 RAPD markers used in this analysis, only seven markers showed any variability within and among these populations, and the Littlefield individuals manifested the majority of this variation. Regardless of this variation, the Littlefield individuals and population clearly grouped with other individuals and sites from the Virgin River drainage and Black Canyon (Fig. 4.3).

**Morphological Analyses**

Principal components analyses of morphological data conducted for each of the sexes were markedly consistent in content; therefore, subsequent results of principal components analyses are with the sexes combined. Five principal components (PCs) that possessed eigenvalues greater than one, explained 74.2% of the overall morphological variance (Table 4.5). The first principal component, accounting for 38.6% of the total variation, had positive loading on all linear measurements and therefore represents overall
size. Bivariate plots of principal components were used to visualize patterns among the designated groups of leopard frogs. The plot of PC II against PC V (Fig. 4.4) exhibited the greatest discriminatory power among groups of leopard frogs and elucidated a difference between leopard frogs from the Virgin River drainage (no *R. pipiens* from upstream locations were included in the analysis) and *R. yavapaiensis* specimens. Principal component II explained 14.9% of the overall variance. Number of thigh bars, number of dorsal spots, number of spots between the eyes, number of spots anterior to eyes, condition of the supralabial stripe, condition of the tympanum spot, amount of chin mottling, and thigh pattern loaded heavily and positively on PC II. PC V explained just 6.0% of the overall variance, but tended to separate leopard frogs from the Imperial Valley from the other groups. Number of dorsal spots, number of eye spots, extent of webbing, and condition of dorsolateral folds loaded positively, while condition of the tympanum spot, condition of the thigh pattern, and condition of the supralabial stripe loaded negatively on PC V.

A minimum convex polygon encompassing all leopard frogs from the Virgin River drainage included only two of the 25 *R. yavapaiensis* specimens (Fig. 4.4). Specimens considered consistent with the description and appearance of the type specimen of *R. onca*, occupied a subset of the morphological variability exhibited by leopard frogs from throughout the Virgin River drainage; but importantly, many leopard frogs from the Overton Arm of Lake Mead and from upstream locations along the Virgin River drainage were similar on bivariate plots to those we considered consistent with *R. onca*. Indeed, although the *R. onca* type specimen scored lower on PC II, several specimens from both the Overton Arm of Lake Mead and from upstream locations had
very similar scores. Leopard frogs from the Imperial Valley generally appeared to be intermediate between frogs from the Virgin River drainage and *R. yavapaiensis*.

Two-way analysis of variance (with interaction) using major species/geographical designation (i.e., all frogs from Virgin River drainage combined, Imperial Valley frogs, and *R. yavapaiensis*) and sex as class variables were conducted for the first five principal components. Interaction terms for all analyses were not significant. Only PC II exhibited significance within the ANOVA model ($F = 24.67, df = 5,77, P = 0.0001$). Species/geographic groups were differentiated by PC II ($F = 58.52, df = 2,77, P = 0.0001$) with *R. yavapaiensis* differing from both Virgin River ($P = 0.0074$) and Imperial Valley frogs ($P = 0.0001$) in pairwise comparisons of least-square means. Leopard frogs from the Virgin River drainage did not differ significantly from the small sample of Imperial Valley frogs ($P = 0.085$). When the Imperial Valley frogs (currently considered *R. yavapaiensis*) were assigned to the *R. yavapaiensis* group, however, a significant difference between leopard frogs from the Virgin River drainage and more southern populations was retained ($F = 100.86, df = 1,79, P = 0.0001$).

**Discussion**

Both mitochondrial and RAPD markers provided a clear signal of historical separation between populations of leopard frogs that occupy the Virgin River drainage and Black Canyon from populations of *R. yavapaiensis* in west-central Arizona and northern Mexico. Furthermore, each genetic marker provided evidence for either identical or very similar genotypes among populations ranging from Littlefield, Arizona downstream to Bighorn Sheep Spring in Black Canyon (Fig. 4.1). Maximum parsimony
and NJ trees produced from mtDNA RFLP data generally supported a monophlectic relationship between *R. yavapaiensis* haplotypes relative to the Virgin River/Black Canyon haplotype within the clade containing both groups. Sequences of mtDNA control region provided a more robust depiction of divergence between Virgin River/Black Canyon samples versus *R. yavapaiensis* in ML, MP and NJ analyses. The difference in degree of resolution between data sets likely derives from both a higher mutation rate in the control region sequence as well as a difference in resolution between indirect (RFLP) and direct (sequencing) protocols. RAPD markers provide a similar and completely congruent indication of separation between Virgin River/Black Canyon populations and *R. yavapaiensis* populations within the total genome (reflecting variation mostly in the nuclear genome).

The hypothesis that leopard frogs from the Virgin River drainage actually represent both *R. onca* and *R. yavapaiensis* populations and possibly their hybrids is inconsistent with the genetic evidence. Congruence between mitochondrial RFLP, control region sequences, and RAPD markers in demonstrating a substantial subdivision of populations into northern (Virgin River/Black Canyon) and southern (*R. yavapaiensis*) lineages argues against the presence of two species or hybridization along the Virgin River drainage and Black Canyon.

Morphologically, leopard frogs from the Virgin River drainage and *R. yavapaiensis* populations appear to exhibit a continuum of multivariate variation with leopard frogs from the Virgin River and *R. yavapaiensis* comprising different ends of the spectrum. That individuals from these two groups of leopard frogs can appear very similar has been the source of much taxonomic confusion. Our morphological analysis,
However, provides quantitative evidence that significant differences exist between the Virgin River leopard frogs and *R. yavapaiensis*. Although the sample size from the Imperial Valley was too small for conclusive analysis, the relationship between the Imperial Valley populations and Virgin River/Black Canyon populations may reflect a common ancestral lineage that evolved along the Colorado River from which all groups are derived. This possibility is supported by the basal position of the Virgin River/Black Canyon leopard frogs on mtDNA trees relative to *R. yavapaiensis* populations (Fig 4.2). When the specimens from the Imperial Valley were forced into the *R. yavapaiensis* group in morphological analyses, however, leopard frogs from the Virgin River drainage remained significantly different from more southern populations.

The concept of Evolutionarily Significant Units (ESU's) provides an objective foundation for delineating conservation units with attention to preservation of evolutionary processes (Moritz 1994a). Under one definition, a group of populations are considered an ESU, regardless of taxonomic designation, if there is substantial evidence of long-term isolation from other populations as determined by a significant phylogenetic structuring of mitochondrial DNA and evidence of substantial divergence in nuclear DNA (Moritz 1994a, 1994b). The genetic patterns observed in this study provide a compelling argument for recognition of leopard frog populations from the Virgin River drainage and Black Canyon as a distinct ESU relative to populations of *R. yavapaiensis* south of Black Canyon. We argue that ESU recognition provides a sound basis for developing conservation management strategies that retain the Virgin River/Black Canyon populations as a separate lineage relative to *R. yavapaiensis*. Other than leopard frogs (purportedly *R. yavapaiensis*) in the Bill Williams River drainage (Trout Creek is in this
drainage system) near the confluence with the Colorado River, we know of no extant populations along the Colorado River south of Black Canyon. We caution, however, that future sampling may show the northern genotype to be established further south along the Colorado River.

The taxonomic history of leopard frogs from the Las Vegas Valley, Virgin River drainage, and adjacent Colorado River is complex, but the genetic data presented here are sufficient to further recognize the leopard frogs from the Virgin River drainage and Black Canyon as a species distinct from more southern *R. yavapaiensis*. The morphological differences between leopard frogs from the Virgin River drainage and *R. yavapaiensis* are consistent with a species level designation between these taxa. Leopard frogs from the Virgin River drainage and Black Canyon should be recognized by the historic name *R. onca* because of the presence of individuals from both extinct and extant populations that match the description and appearance of the type specimen of *R. onca*, and because genetic data allow rejection of the hypothesis that extant populations represent a current introgression of *R. yavapaiensis*.

*Rana onca* populations from the Virgin River/Black Canyon probably represent relatively recent (e.g., late Pleistocene - Holocene) isolates from ancestral populations further to the south. The Virgin River and Black Canyon are in the Mojave Desert and are currently peripheral to areas of the Sonoran Desert occupied by *R. yavapaiensis*. We are uncertain whether *R. onca* populations represent a northern expansion of *R. yavapaiensis*, which then became isolated due to habitat changes caused by fluctuating climatic conditions, or whether *R. onca* represent remnant, northern populations of a
western version of these frogs that once were widely distributed along the Colorado River.

Current evidence suggests that *R. onca* has little genetic diversity within and among extant populations. Given the high level of similarity in all evaluated genetic markers, little information can be derived from our study regarding current gene flow and population structure. A higher-resolution technique might provide the sensitivity required to estimate patterns and rates of gene flow among extant populations, thereby providing a genetic basis for developing conservation strategies beyond recognition of the distributional limits of *R. onca*. Meanwhile, a conservation management plan for the few remnant *R. onca* populations should prioritize the identification of habitat requirements and the reclamation of habitats necessary to maintain population viability. Given our discovery of the extant Black Canyon populations during surveys in 1997 and 1998, it seems prudent to also recommend a more thorough survey of potential habitats within the known range of leopard frogs in the region as well as into adjacent areas along the Colorado River.

**Materials Examined**

Specimens or tissue samples used in each analysis are listed by species, state, county, and site name when available. Alphabetic collection codes follow those listed in Leviton et al. (1985). NK reference samples from the University of New Mexico (MSB). LVT reference samples at the Department of Biological Sciences, University of Nevada, Las Vegas. RDJ represents uncataloged specimens. RDJ samples used in morphological analyses are voucher specimens that have not yet been accessioned at the Barrick
Museum of Natural History, University of Nevada, Las Vegas. Letters following some UMMZ specimens distinguish frogs within a lot assigned a single collection number.

Restriction Site Variation Analysis


Control Region Sequence Analysis

*Rana* spp. (*onca*): Nevada: Clark County: Blue Point Springs LVT 3542; Corral Spring RDJ 925; Rogers Spring LVT 4556-4557; Boy Scout Canyon LVT 3427; Salt Cedar Canyon LVT 3413; Bighorn Sheep Spring LVT 3445; Arizona: Mojave County:
Littlefield LVT 3537-3538. *Rana yavapaiensis*: Arizona: Mojave County: Trout Creek
LVT 4560-4562; Yavapai County: Cottonwood Creek LVT 4566; Tule Creek LVT 4575;
Mexico: Sonora: Sierra San Luis at Rancho Varela NK 3930. *Rana blairi*: New Mexico:
San Miguel County: Conchas River RDJ 899. *Rana berlandiari*: Texas: Brewster
County: Chiricahua Mountains NK 3318. *Rana pipiens*: Nevada: Lincoln County:
Pahranagat Valley LVT 4583.

**RAPD Analysis**

*Rana spp. (onca)*: Nevada: Clark County: Blue Point Springs LVT 3540-3568; Rogers
Spring LVT 4556-4559; Boy Scout Canyon LVT 3426-3428, LVT 3430-3438, LVT
4375-4383; Salt Cedar Canyon LVT 3411-3413, LVT 3467-3470; Bighorn Sheep Spring
LVT 3439-3440, LVT 3442-3453, LVT 4502-4512, LVT 4514-4516; Arizona: Mojave
County: Littlefield LVT 3500-3505, LVT 3533-3539. *Rana yavapaiensis*: Arizona:
Mojave County: Trout Creek LVT 4560-4563, LVT 4578-4581; Yavapai County:
Cottonwood Creek LVT 4565-4574.

**Morphological Analyses**

spp. (onca)*: Nevada: Clark County: Blue Point Spring RDJ 916, RDJ 918-919, 921,
RDJ 929, RDJ 1063; Roger Springs CM 52423, RDJ 922; Corral Spring RDJ 923-927,
RDJ 1062; Muddy River near Glendale LACM 74523. Arizona: Mohave County:
Littlefield LACM 106069, RDJ 1022-23. Utah: Washington County: Berry Spring BYU
9685-9687, BYU 9690-9692, BYU 9696, BYU 9699, BYU 9702-9703; Harrisburg Creek
BYU 12766-12768; near Leeds LACM 106082-106084; near Bloomington BYU 1276,
BYU 1538, LACM 91372-91373, LACM 91375; near St. George BYU 1140, BYU 2782, BYU 12769, CAS 54108-54113, CAS 54117, UMMZ 88543A-B&D. *Rana* spp. (*yavapaiensis*; Imperial Valley): California: Imperial County: LACM 13837, LACM 91311-91312; Riverside County: LACM 91310, LACM 91313. *Rana yavapaiensis*: Arizona: Maricopa County: Cave Creek CAS 17570-17573, CAS 17720, CAS 17722-17724, CAS 17727-17728, CAS 17732, CAS 17735, CAS 20856; Mohave County: near Wikieup LACM 91376; Burro Creek LACM91377-1379; Trout Creek RDJ 1024-1027, 1029-1032.
Literature Cited


Cope, E. D. 1875. *Rana onca*, sp. nov., p. 528-529. In: Dr. H. C. Yarrow, Report upon the collections of Batrachians and Reptiles made in portions of Nevada, Utah, California, Colorado, New Mexico, and Arizona, during the years 1871, 1872, 1873, 1874. Report upon the U. S. Geographical Explorations and Surveys west of the One Hundredth Meridian 5:509-589.


Goebel, A. M., J. M. Donnelly, and M.E. Atz. 1999. PCR primers and amplification methods for 12s ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome b in bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. *Molecular Phylogenetics and Evolution* 11:163-199.


Fig. 4.1. Sample sites for genetic analyses of focal *Rana* spp. (solid circles). Shaded area refers to the mostly contiguous distribution of *Rana yavapaiensis* in the United States (after Platz and Frost 1988); northern distributional limit in Arizona from observations (M. J. Sredl, pers. comm.). Hatched area refers to a historic distribution of putative *R. yavapaiensis* populations centered around the Imperial Valley of California, now possibly be extinct (after Platz 1988).
Fig. 4.2. (A) Maximum parsimony tree of RFLP data under Dollo criteria. *Rana chiricahuensis* was selected as the outgroup. Virgin River/Black Canyon references a single haplotype found in 19 leopard frogs from six sites in the Virgin River drainage and Black Canyon. Bootstrap support is indicated for major clades under both Dollo (first score) and Wagner (second score) parsimony. (B) Maximum likelihood tree generated from control region sequence data under the HKY85 model (Hasegawa et al., 1985). Virgin River/Black Canyon references a single haplotype found in nine leopard frogs from the seven sites in the Virgin River drainage and Black Canyon. *Rana chiricahuensis* was selected as the outgroup. Bootstrap supports are indicated for the same tree generated by maximum parsimony with character state changes unweighted.
Fig. 4.3. (A) Multidimensional scaling (MDS) plot based on pairwise similarity values between individual leopard frogs derived from RAPD data. Individuals from the Virgin River drainage and Black Canyon are indicated by open circles. The polygon encloses all 102 samples from this region. Individuals from west-central Arizona populations of *R. yavapaiensis* are indicated by dark circles. (B) MDS plot based on pairwise similarity values between sample sites, with a minimum spanning network superimposed. Sample sites reference Fig. 4.1 (Littlefield = LF, Blue Point = BP, Rogers = RS, Boy Scout = BS, Salt Cedar = SC, Bighorn = BH, Trout Creek = TC, Cottonwood Creek = CC). Dimension 1 of graph B has been multiplied by -1 for display purposes (only the relative positions of objects are important in MDS configurations and relative position is unchanged by reflection of scales).
Fig. 4.4. Plot of principal component scores for PC II and PC V, derived from morphological data, by species or geographical designation (M = specimens from the Overton Arm of Lake Mead, V = specimens from the Virgin River drainage upstream of Lake Mead, O = specimens from the Virgin River drainage upstream of Lake Mead that matched the appearance of *R. onca*, I = specimens from the Imperial Valley, y = *R. yavapaiensis* from the main distribution in Arizona). The type specimen of *R. onca* is indicated by a star. Lines describe minimum convex polygons for all specimens from the Virgin River drainage (heavy solid line), frogs consistent with the appearance and description of *R. onca* (light solid line), and for *R. yavapaiensis* specimens from western Arizona (broken line). Specimens considered to match the type description and appearance of *R. onca* consisted of the following: BYU 9686, BYU 9691-9692, BYU 9702-9703, BYU 12766-12768, and LACM 106083-106084.
Table 4.1. Primers and polymorphic markers used in RAPD analyses.

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<th>Sequence</th>
<th>Band Scored (base pairs)</th>
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<td>UBC-217</td>
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Table 4.2. Matrix of sequence divergence estimates (Nei and Li, 1979) among haplotypes determined from restriction site variation data. Haplotype 01 references the identical haplotype found in 19 leopard frogs representing six sites from the Virgin River drainage and Black Canyon.

<table>
<thead>
<tr>
<th>Haplotype Number and Taxon</th>
<th>01</th>
<th>02</th>
<th>03</th>
<th>04</th>
<th>05</th>
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<th>09</th>
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<th>12</th>
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<tr>
<td>01 Virgin R./Black C.</td>
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<td>08 <em>R. berlandieri</em></td>
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<tr>
<td>09 <em>R. picipis</em></td>
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<td>12 <em>R. chiricahuensis</em></td>
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Table 4.3. Matrix of Tamura-Nei (1993) distances from control region sequences. Haplotype 01 references the identical sequences from nine individuals representing the seven sample sites within the Virgin River drainage and Black Canyon. Site names for *R. yavapaiensis* haplotypes reference Fig. 4.1. Sample information is listed in the text under Materials Examined. Representative control region sequences are referenced in Genbank by accession numbers AF343776–AF343783.

<table>
<thead>
<tr>
<th>Haplotype Number and Taxon</th>
<th>Samples</th>
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<td>Tule: LVT 4575</td>
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<td>07 <em>R. pipiens</em></td>
<td>LVT 4583</td>
<td>0.2539</td>
<td>0.2604</td>
<td>0.2603</td>
<td>0.2651</td>
<td>0.2471</td>
<td>0.2496</td>
<td></td>
<td></td>
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<tr>
<td>08 <em>R. chiricahuensis</em></td>
<td>LVT 3318</td>
<td>0.2462</td>
<td>0.2567</td>
<td>0.2565</td>
<td>0.2563</td>
<td>0.2364</td>
<td>0.2301</td>
<td>0.1895</td>
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</tr>
</tbody>
</table>
Table 4.4. Pairwise similarity matrix based on RAPD data. The column labeled ‘Within’ provides the average pairwise similarity between samples (Nei and Li, 1979) within a sample site and sample size in parentheses. Following columns present similarity values among sample sites after correcting for average within-site similarities. Sites 1 through 6 are Virgin River and Black Canyon locations, 7 and 8 are *R. yavapaiensis* sites from west-central Arizona. Site names reference Fig. 4.1. Sample information is listed in the text under Materials Examined.

<table>
<thead>
<tr>
<th>Site No. and Location</th>
<th>Site Number</th>
<th>Within (n)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Littlefield</td>
<td>0.9235 (13)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Blue Point</td>
<td>0.9929 (29)</td>
<td>0.9177</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Rogers</td>
<td>0.9559 (4)</td>
<td>0.9388</td>
<td>0.9978</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Boy Scout</td>
<td>0.9870 (21)</td>
<td>0.9777</td>
<td>0.9430</td>
<td>0.9598</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Salt Cedar</td>
<td>0.9638 (7)</td>
<td>0.9476</td>
<td>0.9984</td>
<td>1.0008</td>
<td>0.9726</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Bighorn</td>
<td>0.9898 (28)</td>
<td>0.9433</td>
<td>0.9704</td>
<td>0.9644</td>
<td>0.9761</td>
<td>0.9865</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Trout</td>
<td>0.7295 (8)</td>
<td>0.5267</td>
<td>0.4813</td>
<td>0.4990</td>
<td>0.5123</td>
<td>0.5034</td>
<td>0.4959</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Cottonwood</td>
<td>0.8433 (10)</td>
<td>0.5703</td>
<td>0.5149</td>
<td>0.5348</td>
<td>0.5504</td>
<td>0.5383</td>
<td>0.5317</td>
<td>0.9379</td>
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</tbody>
</table>
Table 4.5. Morphological variables used in principal components analysis and their eigenvector loadings among major principal components (eigenvector loadings with a magnitude less than |0.20| are not shown).

<table>
<thead>
<tr>
<th>Variable</th>
<th>PC I</th>
<th>PC II</th>
<th>PC III</th>
<th>PC IV</th>
<th>PC V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head Width</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head Length</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lip Height</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internarial Distance</td>
<td>0.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tibiofibula Length</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snout-Urostyle Length</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eye Diameter</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tympanum Diameter</td>
<td>0.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsolateral Folds</td>
<td></td>
<td>0.64</td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nose Spots</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eye Spots</td>
<td>0.40</td>
<td>0.31</td>
<td></td>
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</tr>
<tr>
<td>Dorsal Spots</td>
<td>0.44</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thigh Bars</td>
<td>0.47</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thigh Pattern</td>
<td>0.21</td>
<td>0.42</td>
<td>-.32</td>
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<td></td>
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<tr>
<td>Tympanum Spot</td>
<td>0.23</td>
<td></td>
<td>-.63</td>
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<tr>
<td>Lower Lip Mottling</td>
<td></td>
<td>0.27</td>
<td>0.52</td>
<td></td>
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<tr>
<td>Chin Mottling</td>
<td>0.22</td>
<td>-.21</td>
<td>0.56</td>
<td></td>
<td></td>
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<tr>
<td>Supralabial Stripe</td>
<td>0.32</td>
<td>-.35</td>
<td>-.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Webbing</td>
<td></td>
<td>0.54</td>
<td>0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of Variance</td>
<td>38.6%</td>
<td>14.9%</td>
<td>7.7%</td>
<td>7.0%</td>
<td>6.0%</td>
</tr>
</tbody>
</table>
Appendix I. Descriptions of continuous morphological characters (1-8) and coding protocols for discontinuous morphological characters (9-19) used in morphological analyses.

1. Head width: at mid tympanum on the jaw.
2. Head length: from the angle of the jaw to the tip of the snout.
3. Lip height: from the ventral margin of the upper lip to the nostril.
4. Internarial distance: from nostril to nostril.
5. Tibiofibula length: from knee to ankle gently pressing soft tissue.
6. Body length: from the urostyle to the snout.
7. Tympanum diameter: measured at its greatest diameter.
8. Eye diameter: measured at its greatest diameter.
9. Number of spots anterior to eyes: a count of dorsal spots anterior to the eyes.
10. Number of spots between eyes: a count of the spots on top of and between the eyes.
11. Number of dorsal spots: a count of spots behind the eyes and between dorsolateral folds.
12. Number of transverse bars: a count of the bars or spots on the dorsal surface of thigh.
13. Condition of the dorsolateral folds: 1 = continuous folds, 2 = broken posteriorly, 3 = broken and inset medially, 4 = not well defined posterior to break.
14. Pattern on the posterior surface of the thigh: 1 = immaculate, 2 = spotted, 3 = obscurely (fussily) reticulated, 4 = distinctively reticulated, 5 = white spots on a dark field, 6 = coalescing white spots or a white reticulation on a dark field, 7 = obscurely dark, fuscous with no discernible pattern.
15. Melanite pigment on lower lip: 1 = conspicuously mottled, 2 = obscurely mottled, 3 = flecked, 4 = suffused, 5 = immaculate.
16. Melanite pigment on the chin: character states as for melanite pigment on lower lip.
17. Condition of the tympanum spot: 1 = absent, 2 = faint or obscure, 3 = conspicuous
18. Condition of the supralabial stripe: 1 = absent, 2 = well defined posterior to the eye, 3 = present posterior and anterior to the eye but not conspicuous, 4 = as in 3 but conspicuous.
19. Condition of webbing on fourth toe: 1 = extending onto terminal phalange, 2 = to the distal tip of subterminal phalange, 3 = about midway along the subterminal phalange, 4 = to the distal tip of third phalange, 5 = about midway along the third phalange.
Appendix II. Restriction site variation data with haplotypes. Restriction sites are coded as binary data. Haplotype 01 references the identical haplotype found in 19 leopard frogs from 6 sites in the Virgin River drainage and Black Canyon. Site data indicated for *R. yavapaiensis* haplotypes reference Fig. 4.1. Sample information is listed in text under Materials Examined.

<table>
<thead>
<tr>
<th>Haplotype No. and Taxon Designation</th>
<th>n</th>
<th>Samples</th>
<th>Bsp</th>
<th>Bst</th>
<th>Hae</th>
<th>Hha</th>
<th>Hinc</th>
<th>Hinf</th>
<th>Dpn</th>
<th>Msp</th>
<th>Rsal</th>
<th>Sau</th>
<th>sTaq</th>
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</thead>
<tbody>
<tr>
<td>01 Virgin R./Black C.</td>
<td>19</td>
<td>All Samples</td>
<td>11</td>
<td>110</td>
<td>111101110</td>
<td>010</td>
<td>10101</td>
<td>001000</td>
<td>001110000</td>
<td>10100101</td>
<td>110</td>
<td>11100100</td>
<td>1111</td>
</tr>
<tr>
<td>02 <em>R. yavapaiensis</em></td>
<td>1</td>
<td>Trout: RDJ 1024</td>
<td>01</td>
<td>110</td>
<td>111011100</td>
<td>000</td>
<td>00101</td>
<td>001000</td>
<td>001110000</td>
<td>10000101</td>
<td>110</td>
<td>11100110</td>
<td>1111</td>
</tr>
<tr>
<td>03 <em>R. yavapaiensis</em></td>
<td>2</td>
<td>Trout: RDJ 1026,1029</td>
<td>01</td>
<td>110</td>
<td>111101101</td>
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<td>00101</td>
<td>001000</td>
<td>001110000</td>
<td>10000101</td>
<td>110</td>
<td>10100110</td>
<td>1111</td>
</tr>
<tr>
<td>04 <em>R. yavapaiensis</em></td>
<td>1</td>
<td>Trout: RDJ 1028</td>
<td>01</td>
<td>110</td>
<td>111101101</td>
<td>000</td>
<td>00101</td>
<td>001000</td>
<td>001110000</td>
<td>10000101</td>
<td>110</td>
<td>10100110</td>
<td>1111</td>
</tr>
<tr>
<td>05 <em>R. yavapaiensis</em></td>
<td>2</td>
<td>Tule: RDJ 1205,1208</td>
<td>01</td>
<td>110</td>
<td>111101101</td>
<td>000</td>
<td>00101</td>
<td>001000</td>
<td>001110000</td>
<td>10000101</td>
<td>110</td>
<td>11100110</td>
<td>1111</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>R. Varela: NK 3929,3933</td>
<td>01</td>
<td>010</td>
<td>111101101</td>
<td>000</td>
<td>00101</td>
<td>001000</td>
<td>001110000</td>
<td>10000101</td>
<td>110</td>
<td>11100110</td>
<td>1111</td>
</tr>
<tr>
<td>06 <em>R. yavapaiensis</em></td>
<td>3</td>
<td>R. Varela: NK 3926,3927,3930</td>
<td>01</td>
<td>010</td>
<td>111101101</td>
<td>000</td>
<td>00101</td>
<td>001000</td>
<td>001110000</td>
<td>10000101</td>
<td>110</td>
<td>11100110</td>
<td>1111</td>
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<tr>
<td>07 <em>R. berlandieri</em></td>
<td>1</td>
<td>LVT 4564</td>
<td>01</td>
<td>100</td>
<td>010101110</td>
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<td>000</td>
<td>101111010</td>
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<td>08 <em>R. berlandieri</em></td>
<td>1</td>
<td>LVT 4594</td>
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<td>110101100</td>
<td>000</td>
<td>101111010</td>
<td>0111</td>
</tr>
<tr>
<td>09 <em>R. pipiens</em></td>
<td>7</td>
<td>All Samples (except RDJ 903)</td>
<td>01</td>
<td>001</td>
<td>110111111</td>
<td>110</td>
<td>01111</td>
<td>0110100</td>
<td>111010110</td>
<td>110111000</td>
<td>000</td>
<td>01101100</td>
<td>0010</td>
</tr>
<tr>
<td>10 <em>R. pipiens</em></td>
<td>1</td>
<td>RDJ 903</td>
<td>01</td>
<td>001</td>
<td>110111111</td>
<td>110</td>
<td>01111</td>
<td>0110100</td>
<td>111010110</td>
<td>110101100</td>
<td>000</td>
<td>01101100</td>
<td>0010</td>
</tr>
<tr>
<td>11 <em>R. blairi</em></td>
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<td>000</td>
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<td>000</td>
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<td>1111110</td>
<td>011010000</td>
<td>110101100</td>
<td>000</td>
<td>111111100</td>
<td>0111</td>
</tr>
<tr>
<td>12 <em>R. chiricahuensis</em></td>
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<td>missing</td>
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<td>missing</td>
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<td>001</td>
<td>011010101</td>
<td>0011</td>
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</tbody>
</table>

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Inhabiting the Mojave Desert

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